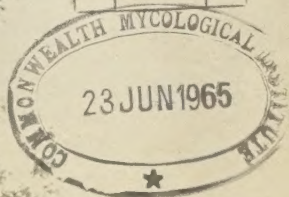


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Verticillium Wilt of Maples

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VERTICILLIUM WILT OF MAPLES

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Deep appreciation is expressed to Dr. Frank L. Howard through whose efforts this publication has been possible.

Cover: A 24-year-old sugar maple tree showing wilt in all sections of the crown.

The material in this technical bulletin is a resumé of a thesis submitted by the author as a partial fulfillment for the degree of Doctor of Philosophy at Brown University in June 1954. Contribution 915 of the Rhode Island Agricultural Experiment Station.

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INTRODUCTION

The subject of the investigations reported in this technical bulletin concerns a vascular disease or Tracheomycosis of maple trees, known as "maple wilt" and incited by a species of the fungus, *Verticillium*. This fungus has also been recognized as the cause of serious diseases of numerous other economic woody and herbaceous plants. In fact, this pathogen has been reported to cause diseases of plants, growing especially in the temperate climate of the world and belonging to at least 18 orders, 38 families, 96 genera and 136 species.

Maple wilt was first reported by Rankin (1914) who detected the disease affecting hard maples growing near Claverack, New York. The trouble was attributed to a fungus belonging to the genus *Acrostolagmus* which was later determined to be a species of *Verticillium* by Zimm (1918). Between 1914 and 1926 the disease had become so commonly noticed that Gravatt (1926) published a circular which reported that the disease was affecting trees growing in various localities from Eastern Canada and Wisconsin to the Carolinas. Within the past two decades maple wilt has become increasingly prevalent on maple trees growing in parks, on private grounds and along streets. Nevertheless, a detailed study of various aspects of the disease based on experimentation has been neglected, perhaps because of the complexity of the problem or because of the seemingly insignificant economic importance of the disease.

Therefore, the investigations reported here were undertaken for the purpose of gaining as broad an understanding as possible of the fungus mycologically, culturally and physiologically, and of the *modus operandi* of the disease, with particular emphasis upon the factors influencing the initiation of the disease and its pathogenicity and the nature and properties of the toxic metabolite produced by the fungus. From the knowledge obtained, it is hoped that information will be available to assist in formulating a technically sound and economically feasible program of control.

THE DISEASE

Maple wilt can be recognized by the wilting and dying of leaves on one or more branches (plate 1). Two types of symptoms have been observed: (1) *chronic*, in which wilt generally appears on one branch and then progresses slowly to other parts of the tree during the same or several subsequent seasons; and (2) *acute* where the entire tree is affected within a period of a few weeks. In either case, wilted leaves become dry and may fall prematurely, or remain attached to dead branches. Internal symptoms are evident as bluish or blackish-green streaks in the sapwood (Plate 2). Streaks may be limited to a single affected branch or they may extend from the roots to a branch displaying foliar symptoms.

Little information is available in the literature on the conditions influencing

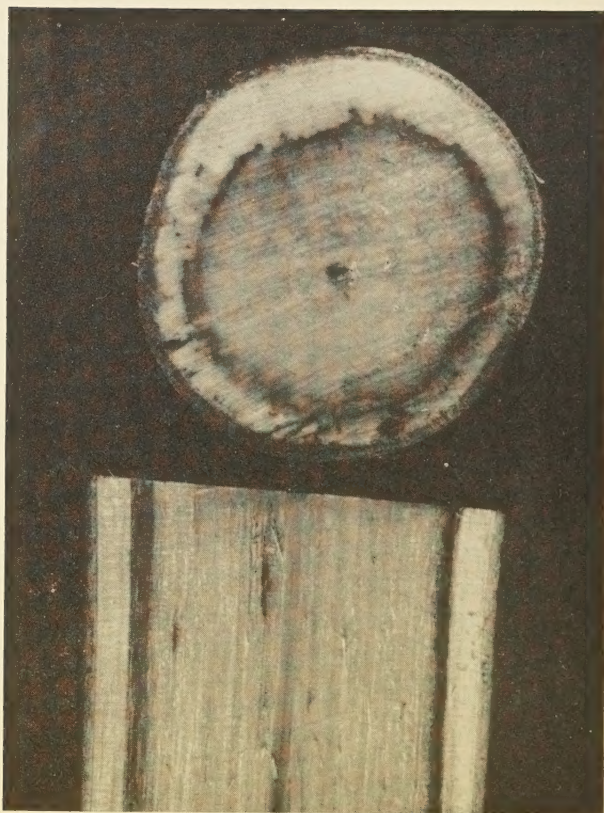


Plate 2—Radial and transverse sections of a sugar maple tree exhibiting extensive discoloration of the sapwood. Inoculum was introduced at 3 different points on the trunk.

the initiation of the maple wilt disease and the pathogenicity of the causal organism. Numerous factors considered responsible for these conditions have been based upon casual observations, circumstantial evidence, or upon the activity of the *Verticillium* fungus as it affects unrelated hosts rather than upon experiments using maple trees. Arborists have often reported that maple wilt appears to be prevalent following periods of drought. The prevailing opinion among these observers is that drought predisposes trees to injury by the fungus. Based upon experience with other *Verticillium*-infected plants, pathologists feel that there is a relationship between the amount of soil moisture and the incidence of disease. However, their opinions vary. It has been assumed by many workers that since *Verticillium* is a soil-inhabiting fungus it must obviously spread through the soil

to trees which can be invaded via the root system. Arborists have also speculated that the maple wilt fungus might possibly be spread by insects which infest the foliage or other parts of maple trees. It has been noted that following severe wind storms maple wilt becomes increasingly prevalent especially among ornamental trees. Two schools of thought exist regarding the reason for such outbreaks: one contends that the fungus can readily infect trees via the roots which were injured as the wind whipped the crown of these trees; the second is of the opinion that because of an injured root system trees become devitalized and thus more susceptible to disease. The latter belief has added impetus to the practice of fertilizing or "feeding" trees for the control of maple wilt.

In order to test the validity of these observations, it was necessary to conduct a variety of experiments upon potted seedlings and naturally seeded field-grown sugar maple (*Acer saccharum* Marsh).

The records of infection were taken on the extent of sapwood discoloration in some cases and degree of wilt in others. Extensive greenish discoloration of the sapwood usually accompanies the initiation of the disease, but localized discolored areas may occur near wounds in control trees and in connection with other injuries. In all cases, the use of discoloration as a criterion of pathogenicity or as evidence of the spread of the fungus within the tree was validated by positive diagnosis of cultures of the fungus isolated from the discolored areas. Wilt symptoms of potted seedlings and small, field-grown trees have been estimated in percentage of the entire tree infected. On large trees, wilt was scored as 1, 2, or 3, accordingly as the wilt appeared in the lower third, in the lower two-thirds, or in the entire tree, respectively.

Little mention is made of the extent of die-back of twigs, because the degree of such injury was noted to be closely correlated with the percentage of wilt of the foliage.

For the inoculation experiments, the only pathogenic isolate used was our V-10, which was isolated from a severely infected Norway maple (Plate 10).

Factors Influencing the Experimental Initiation of Disease in Maple Trees

METHODS OF INOCULATION

In order to facilitate investigations of various phases of the maple wilt disease and to gain some knowledge of the conditions affecting the initiation of the disease, it was first necessary to develop efficient and practical methods of inoculation. Two direct methods of trunk inoculation, which are modifications of techniques used by various workers (Banfield, 1938, 1941; Zentmyer, 1946; Caroselli and Feldman, 1951) to test the pathogenicity of other fungi, were employed with satisfactory results. In all cases, the inoculum was taken from colonies which were similar in appearance and displaying no sectoring. Colonies selected for this purpose had

been grown from mass transfers from the marginal portions of a typical isolate of strain V-10.

The Agar-Disc Method

This method consisted of inserting a disc of agar containing an actively growing colony of the fungus into a vertical wound made by a knife or scalpel

Table 1 — Relation of Inoculation of Bark and Sapwood to Initiation of Disease in Field-Grown Trees.

No. of Trees	DBH of Trees (Inches)	Height Range (Feet)	Date Inoculated	Place of Inoculation	No. Trees with Green Discolored Wood	No. Trees with Wilt	No. Trees Positively Diagnosed
13	2-5	8-15	8-10-50	Bark	0	0	
37	2.5-5	8-19	8-10-50	Sapwood	34 (92%)	30 (81%)	30 (81%)

sterilized in alcohol. Usually, the inoculum consisted of spores, microsclerotia and mycelium. Drying out of the inoculum was prevented by a covering of wet absorbent cotton secured by a strip of adhesive tape. By experiment, it was found that successful inoculation necessitated incision into the elements of the sapwood. In 37 trees, the incision was made deep enough for the inoculum to come in contact with the severed vessel elements and in 13 trees the inoculum was applied to gently scraped outer bark. Whereas 92 percent of the first group exhibited streaking of the sapwood and 81 percent displayed wilt symptoms, none of the bark-inoculated trees developed symptoms of the disease, as shown in Table 1.

These experimental results are significant since there are reports in the literature that this fungus affects the cortical tissue of maple trees. For example, Gravatt (1926) associated bark-lesions and slime-flux with maple wilt. Rudolph (1926) found the cortical tissues of herbaceous plants to be infected with *Verticillium*. The present writer, on the contrary, has never been able to connect such bark abnormalities with the wilt disease nor to attribute them to the fungus in question, but he found that bleeding lesions on such trees were associated with *Phytophthora cactorum* (Leb & Cohn) Schroet.

The Spore-Suspension Method

A spore suspension, prepared by gently stirring ten ml. of sterile, distilled water on the surface of a sporulating mycelial mat growing on malt agar, was introduced into small trees by the following technique. The split end of a 2-inch length of rubber tubing placed over the area to be inoculated and made secure by wire was wrapped with non-injurious plastic putty (Plate 3). After pipetting about 1 ml. of the spore suspension into the tubing, a wound was made by inserting a sharp instrument down the rubber tube.

Inoculation of large field trees was accomplished by introducing 5 ml. of the spore suspension into holes bored into the sapwood through an apparatus of rubber and glass tubing fitted with a bored cork. One trunk inoculation was made in each tree. After the suspension had been absorbed by the tree, the hole was

plugged with a solid wooden dowel. It was found that trunk inoculations made about 6 inches above the soil were more effective for studies of the disease than those made higher up the tree.

All twig inoculations mentioned in experiments throughout this paper were made with spore suspensions in the following manner: the twigs were held in a



Plate 3—Two potted sugar maple seedlings inoculated by the agar-disc method (left), and the spore-suspension method (right).

horizontal position and a small cylinder of plastic putty was attached vertically. A cut was then made into the xylem with a sharp knife or scalpel through the spore suspension in this plastic container. It was found that the inoculum was absorbed by the tree almost immediately.

Root inoculations were made in the manner described for the agar disc method, except that the inoculated area was covered with soil instead of wet cotton.

A Comparison of the Agar-Disc and Spore-Suspension Methods

The relative effectiveness of these two methods was determined by separately inoculating 2 groups of 10 potted trees each, on June 12, 1951. As checks, 5 trees were inoculated with sterile malt agar and 5 with water which had previously been in contact with sterile medium. Although the percentage of initial infection

Table 2 — Relative Effectiveness of Spore-Suspension and Agar-Disc Inoculation of Potted 2-Year-Old Sugar Maple Trees.

No. of Trees	Date Inoculated	Inoculation Method	Seasonal Development of Symptoms							
			7-2-51		8-4-51		8-26-51		9-1-51	
			No. Trees	% Wilt	No. Trees	% Wilt	No. Trees	% Wilt	No. Trees	% Wilt
10	6-12	Spore Susp.	4	60*	6	70	8	80	8	80
10	6-12	Agar Disc	3	50	5	50	7	70	8	80
5	Control		0	0	0	0	0	0	0	0
5	Control		0	0	0	0	0	0	0	0

*Wilt expressed as average wilt of infected trees.

Table 3 — Relative Effectiveness of Spore-Suspension and Agar-Disc Inoculation Methods when Applied to the Root of Large Field Trees.

No. of Trees	DBH of Trees (Inches)	Date Inoculated	Inoculation Method	Average Wilt of Trees in Each Group			
				12 Aug.	24 Aug.	31 Aug.	4 Sept.
10	7/8-3	6-13-52	Agar Disc	0.8	1.3	1.1	1.6
10	3/4-3	6-13-52	Spore Susp.	0.5	0.8	1.3	1.4
5	1-3			0	0	0	0

was higher and the progress of the disease was more rapid in plants inoculated by the spore-suspension method, there was no difference between these two groups at the end of the season (Table 2). On September 1, 1951, 80 percent of the trees in both groups exhibited wilt symptoms. Although there was no difference in severity of disease when either of these methods was used, the spore-suspension method was the one chosen for subsequent experiments, because trees could be inoculated with greater dispatch and facility.

A comparison of these two methods was also made for root inoculation of large field grown trees. Again there was little difference (Table 3).

DATE OF INOCULATION

Inoculations were made at various times during the growing season in an effort to obtain some information as to the time of year when trees are most susceptible to the disease. Field trees, inoculated by the agar disc method, were divided into 5 groups and 1 group was inoculated on each of the following dates in 1951: May 14, June 14, July 18, August 1 and August 31. Progress of the disease, recorded throughout the course of the experiment which terminated on September 1, was determined by the extensiveness of the wilt according to the score system previously described.

Results of these tests are shown in Table 4 and expressed graphically in Figure 1.

Figures representing wilt are averages of all the trees affected. The most severe wilt symptoms were noted on trees inoculated on May 14 and June 14. Trees inoculated later in the season exhibited less pronounced symptoms.

Table 4 — Relation of Date of Inoculation to Initiation of Disease in Field Trees.

No. of Trees	Date of Inoculation*	Type of Inoculation	Average Degree of Wilt of all Trees			
			6-30-51	7-15-51	8-20-51	10-1-51
6	5-14-51	Agar Disc	1.0	1.5	2.1	2.9
5	6-14-51	Agar Disc	0.9	1.2	1.9	2.9
6	7-18-51	Agar Disc	—	—	0.3	0.9
7	8- 1-51	Agar Disc	—	—	0	0.1
6	8-31-51	Agar Disc	—	—	—	0
5	Control	Agar Disc	0	0	0	0

*One tree inoculated on each of above dates with sterile medium.

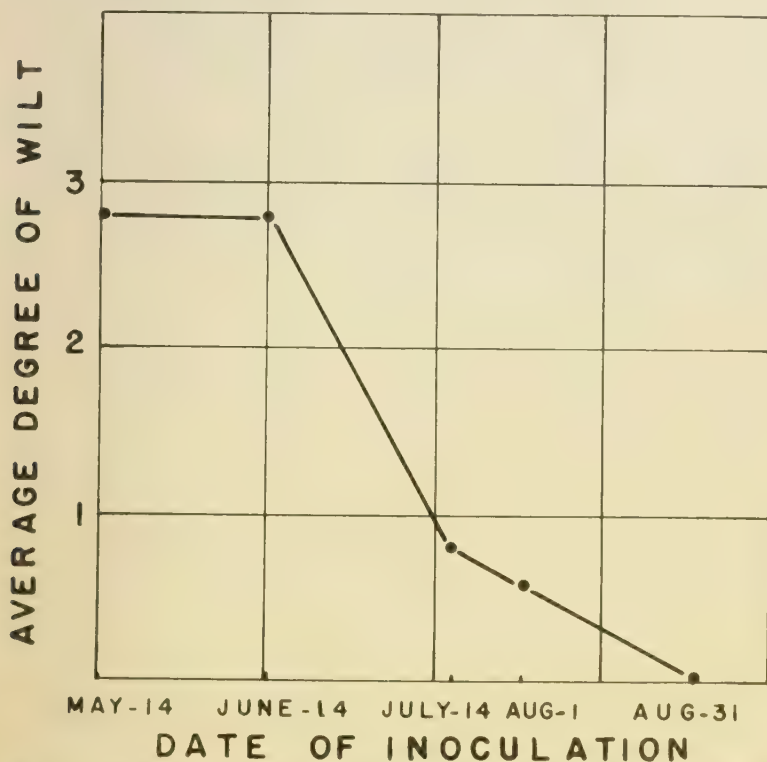


Figure 1—Effect of time of inoculation on the development of the maple-wilt disease.

Many factors may be responsible for the difference in initiation or progress of the disease. A few of the more probable ones are given brief consideration

That a longer incubation period is responsible for the more pronounced disease symptoms does not appear valid, since trees inoculated on June 14 showed the same degree of wilt as the trees inoculated on May 14. Furthermore, it was observed that in the small trees in this group the maximum wilt occurred within 5 weeks after both the May and June inoculations. This was not the case with trees inoculated later in the season.

Whether there is a difference in chemical constitution or contents of the springwood and the summerwood was not determined. It was noted, however, that a decoction medium made from young, succulent twigs supported a much better growth of the fungus in the laboratory than did a medium made from chips of wood with 3 or 4 annual rings.

Differences in the initiation of disease may be the result of weather and temperature variations. The warm weather of July and August may have had some effect upon the viability of the inoculum. It was reported by McKay (1921) and Rudolph (1926) that the optimum temperature for development of the *Verticillium* wilt in potatoes and tomatoes is about 21°C., with the maximum temperature 24°C. Ludbrook (1933), experimenting with herbaceous plants growing in temperature-controlled soil infested with *Verticillium albo-atrum* Reinke and Berth., found that infection was slight at 82°F. (28°C.) and none at 86°F. (30°C.). Similar temperature-growth relations were reported by McKeen (1943) for tomatoes, by Schneider (1948) for guayule and by Edson and Shapovalov (1920) for eggplant.

In the temperature studies reported here under the physiology of *Verticillium* sp., it will be noted that under laboratory conditions the optimum growth of 3 strains, which were representative of 10 strains of this fungus, was between 20° and 23°C., with no growth occurring above 30°C.

Whether there is a relationship between the carbohydrate reserve and the incidence of disease was not determined, but deserves some attention, since the disease pattern is similar to that of the Dutch elm disease, which was found to be correlated with the carbohydrate reserve by Feldman, Caroselli and Howard (1949-50). When the carbohydrate reserve in elm trees was at a low level immediately after full leaf, Feldman et al. (1950a) were able to suppress wilt symptoms by spraying leaves with sugar solutions.

EFFECT OF TRUNK AND TWIG INOCULATION

Since it was shown previously that the disease is artificially induced in woody stems only when the inoculum comes in contact with the severed vessels, it was to be expected that the disease would progress upward in the tree and not downward to any great extent. This upward spread is in accord with everyday observations of the *Verticillium* wilt in the field—a situation quite the opposite of that in the progressive development of the Dutch elm disease—but as far as the writer knows,

there has never been any experimental demonstration of this observation. For this purpose, therefore, inoculations were made in the trunks of 10 trees, 1 to 6 inches DBH and on 4 twigs on each of 10 other trees. Five check trees were used for each test.

Table 5 — Comparison of Trunk and Twig-Inoculation in Disease Development.

No. of Trees	Date of Inoculation	Point of Inoculation	No. of Inoculations Per Tree	No. of Diseased Trees	No. of Trunk-Inoculated Trees Affected by Degree of Wilt			No. Diseased Branches Twig-Inoculated
					1°	2°	3°	
10	5-21-51	Trunk	1	9	2	1	6	—
10	5-21-51	Twig	4	10	10	0	10*	36
5	Control	0	0	0	0	0	0	0
5	Control	0	0	0	0	0	0	0

*Twigs were selected from the upper part of the tree.

The results, as listed in Table 5, indicate greater spread of the disease in the trees inoculated in the trunk than in the twigs. Disease symptoms in the latter were confined to the inoculated twigs alone. These were wilted and the streaking was mostly upward, with very little downward, and no spread of the disease to other twigs. In the trunk inoculated trees, wilt developed in all but one and in most cases was severe. Again, there was little green discoloration downward; the upward extent of the discoloration was determined for some distance but with no idea of obtaining the complete extent. All check trees remained uninfected.

Since it was clearly demonstrated that the spread of this fungus is upward in inoculated, field-grown trees, there can be no question that the progress of the disease will be the same under conditions of natural infection.

The foregoing has an important bearing on certain control methods, especially the matter of pruning and the possibility of spreading infection by means of pruning tools. Gravatt (1926) was of the opinion that the maple wilt is spread by means of these implements and recommended the sterilization of them and the treatment of wounds with a coal tar-creosote mixture.

If, however, one keeps in mind that the maple wilt fungus progresses only upward in the tree and that therefore an infection at a pruning cut could be only local, then pruning tools, whether clean or not, are not responsible for the spread of the disease, because branches are severed. Under the conditions of the following experiment, it is shown that pruning tools do not transfer the fungus.

Each of 10 field trees 2 to 8 inches DBH were wounded at 4 different places on the trunk with a saw that was first used to cut through a 2-inch piece of infected wood before each wound was made. At the end of the season, all 40 wounds had healed completely and were free of any injury by the fungus.

EFFECT OF THE NUMBER OF INOCULATION POINTS ON THE INCIDENCE OF DISEASE

It was demonstrated throughout the author's work with *Fusicillium* that

90 percent of young, inoculated, potted trees will succumb after one inoculation and that the percentage of success with larger field trees is rarely this high. It was conjectured that distribution of the fungus inoculum over a wider area of the sapwood might result in more extensive expression of the wilt. This idea was tested by varying the number of inoculation points on field-grown trees using the spore-suspension method. Each tree in 4 groups of 5 trees each was inoculated in the trunk at 2, 3, 4, and 5 points. Two additional trees for each group were selected as checks.

Table 6 — Effect of Number of Inoculation Points in Trunk on Spread of Disease in Sugar Maple Trees Inoculated May 24, 1951.

No. of Trees	Type of Inoculation	No. of Inoculation Points	No. Trees Exhibiting Wilt	Average Degree of Wilt	No. of Trees Showing Severe Wilt	Complete Cylinder of Sapwood Discoloration in Trunk	
						No. Trees Examined	No. Trees Affected
5	Spore Susp.	5	5	2.9	1	2	2
5	Spore Susp.	4	5	2.9	1	2	2
5	Spore Susp.	3	5	2.9	1	2	2
5	Spore Susp.	2	5	3.0	1	2	2
2	Control	—	0	0	0	2	0
2	Control	—	0	0	0	2	0
2	Control	—	0	0	0	2	0
2	Control	—	0	0	0	2	0

As indicated by the results presented in Table 6, there was no difference in the degree of wilt regardless of the number of inoculation points. One tree in each group exhibited severe wilt symptoms throughout the season and these were dead the following year. The trunks of two trees in each group, when cut crosswise in several places, disclosed a complete ring of sapwood discoloration extending upward from the points of inoculation. Although this condition rarely occurred in trees inoculated at one point, such trees generally did not display a much lower degree of wilt than did multi-inoculated trees. This failed to support the belief that *Verticillium* wilt results from the plugging of the vessels.

EFFECT OF SPORE LOAD

Spores produced by certain fungi are one phase of the life cycle that afford an opportunity for quantitative appraisal. Snell (1942) clearly demonstrated a definite threshold or quantum relationship between the sporidium production of *Cronartium ribicola* and infection of white pine. A quantitative correlation between the number of spores on a single wheat seed and infection of that seed by the bunt organism, *Tilletia tritici* (Bjerk.) Wint., was demonstrated by Heald (1921). Therefore, the ability of the maple wilt fungus to produce an abundance of spores on artificial media stimulated interest as to the possible inoculum potential of the spore *per se*.

The trunk of each tree in 5 groups of 5 trees each was inoculated with a

spore load of 10 , 10^2 , 10^3 , 10^4 , and 10^5 . Two additional trees were used as checks. The numbers of spores were determined by averages from 10 microscopic fields of a given dilution. The lower dosages were attained by serial dilutions. In an effort to reduce the quantity of mycelial fragments, the spore suspension was developed in 500 cc. Erlenmeyer flasks which were set up in the manner of chemical wash bottles and in which the fungus was grown on a sterile, modified, Tochinai liquid medium. After incubation of about 3 weeks, with slight agitation, the spore suspension in the liquid was forced out by blowing air into one of the glass tubes.

Table 7 — Effect of Spore-Load on Incidence of Disease from Trunk Inoculation on May 14, 1952, and Recorded September 4, 1952.

No. of Trees	Spore Load	No. of Trees Exhibiting Wilt	Range of Wilt 4-9-52 (%)	Average Wilt of 5 Trees (%)
5	10	1	0-10	2
5	10^2	1	0-10	10
5	10^3	3	0-50	24
5	10^4	4	0-60	39
5	10^5	5	30-70	54

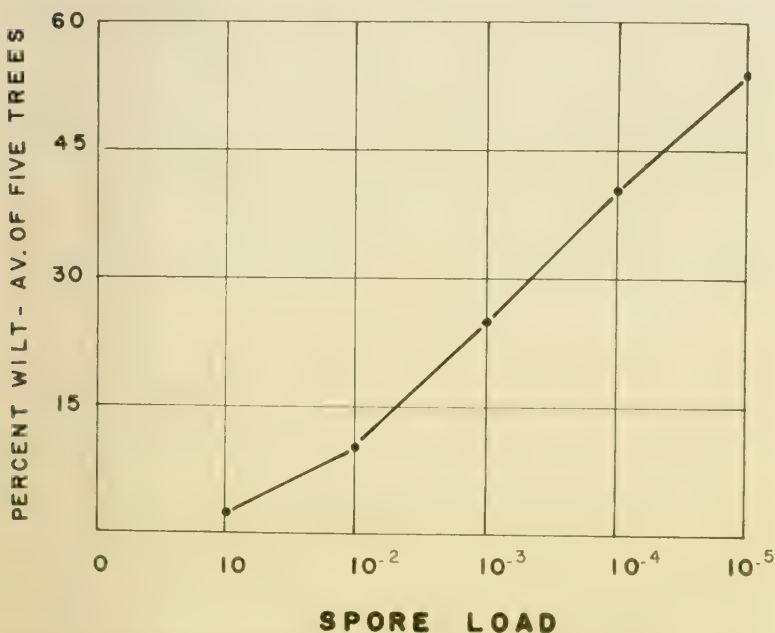


Figure 2—The effects of spore load on percentage of wilt.

The results, as presented in Table 7 and expressed graphically in Figure 2, indicate an increase in severity of disease with an increase of spore load. Although no work has been done by the writer on the vigor or germinating properties of *Verticillium* conidia, it appears that infection is probably dependent upon the number of viable conidia rather than the number of spores introduced.

EFFECT OF A COMBINATION OF CULTURE FILTRATE AND SPORE SUSPENSION UPON THE INCIDENCE OF DISEASE

It has been demonstrated that spores alone will produce disease in both potted and larger, naturally growing trees (up to 6 inches in diameter). Later in this bulletin it will be demonstrated that toxin produced by the fungus will bring about disease in potted trees. It was deemed desirable to test a combination of spore suspension and toxin for severity of wilt produced in trees $\frac{1}{2}$ to 2 inches DBH. This testing was begun on June 23, 1952, using the toxin at the rate of 50 cc. per inch of DBH.

The results recorded in Table 8 show that the toxin-spore mixture produced more severe wilt symptoms than the spore suspension alone. Also, it appears that the more severe initial symptoms of wilt on trees given the combination inoculum may be attributed to the presence of the toxin.

Table 8 — Effect of Spore Suspension, and Spore Suspension plus Culture Filtrate on Progress of the Disease in Sugar Maple Trees $\frac{1}{2}$ " to 1 $\frac{1}{4}$ " DBH.

No. of Trees	Type of Inoculation	Date of Inoculation	Average % of Wilt		
			7-31-52	8-4-52	8-29-52
10	Spore Suspension and Toxin	6-23-52	27	41	70
10	Spore Suspension	6-23-52	6	25	25
5	Controls	—	—	—	—

FOLIAR INOCULATIONS

Rankin (1914) detected microsclerotia and conidia of *Verticillium* in the xylem elements of leaves as well as of the stems of maple trees. Even though there can be no doubt that the fungus can penetrate the leaves from the stem tissues below and will normally do so at least to a limited extent, the question naturally arises as to whether or not the fungus can invade the leaves directly from the outside.

An experiment employing 19 potted sugar maples and 1 Norway maple was designed to test this possibility. The leaves of 10 trees were pricked with a needle on and between the veins, 10 punctures to each leaf. The leaves of 5 trees were atomized with a heavy spore suspension, and the leaves of 5 more with media washings. In addition, two groups of five trees with unwounded leaves were

similarly sprayed, as controls. None of these trees showed any symptoms of wilt throughout the growing season.

In order to detect the fungus in the leaves at the end of the experiment, two leaves selected at random from each plant were removed and surface-sterilized, and pieces of petiole and blade were planted on sterile malt agar. In no instance was the fungus isolated from any of the portions of leaves, blades, or petioles. Under the conditions of the experiment, it seems unlikely that invasion of maple trees by this fungus occurs by way of the leaf tissues.

SOIL INOCULATION STUDIES

The methods of spread of the maple wilt fungus have long interested arborists and forest pathologists. Dissemination by means of pruning tools has been discussed above. Other agents suggested have been such birds as sapsuckers and such insects as the maple-twig pruners. Furthermore, observations on the pattern of spread suggest dissemination through the soil. Two examples are particularly striking.

The first case concerns a group of sugar maple trees planted about 25 years ago in 2-row plots of 10 trees each about 5 feet apart each way. The entire area sloped so that soil drainage and runoff were in an easterly direction. Wilt was noted in the summer of 1947 on one of the trees growing at the extreme upper end of the plot. The disease in this tree progressed in 1948 and by 1949 the tree was so badly affected that it was removed (Plate 1). In August 1949, the next tree in line downhill exhibited symptoms of wilt, and in July 1953, the third tree down was affected. Cultures made from transplants taken from discolored areas in the trunk of this tree yielded the causal agent, *Fusicoccum* sp. Although all the trees in these plots had been pruned in 1949, it seems unlikely that this practice was responsible for the spread of the disease, since an infection court would have been detectable at the wounded areas made by the pruning implements. Furthermore, the lower trees were pruned first.

The second case concerns a row of Norway maple trees in a cemetery located in New York City. Samples taken at random from three of these trees were cultured and *Fusicoccum* sp. was isolated. According to the superintendent, these trees had been transplanted as 5- or 6-year-old saplings about 40 years before the inspection, which was made in July 1953. These trees had been planted approximately 20 feet apart in an area with a definite slope of about 2 to 3 percent grade. One side of the root area of the row of trees was covered by a macadam roadway that had been constructed about 1925, whereas the other side was lawn that was rarely disturbed because it was an old burial plot. According to the information available, these trees had not been pruned for at least three decades, and the trunks, with the exception of one tree which appeared to have been recently scarred, were free of wounds. The pattern of spread of the disease in

these trees was similar to that observed in case one. Two trees at the top of the slope had been removed because they were severely affected. Characteristic symptoms of the disease, as indicated by the dead and dying branches, sparseness and yellowing of the foliage, occasional branches with wilting leaves, and staghead appearance of the large, scaffold branches were decreasingly severe on the trees more remote from the trees which had been removed originally. It appeared that the spread of the fungus from one tree to another had taken place through the soil in a downhill direction. It may be added parenthetically that horizontal distribution of *Ferticillium albo-atrum* in 20 different localities, regardless of soil type, past crop history of the land, and climatic environments, has been reported by Wilhelm (1950).

A review of literature indicates that most observers feel that this disease is contracted through the root system of susceptible hosts. Reinke and Berthold (1879), the first definitely to establish the etiology of this disease, demonstrated the ability of the fungus to invade apparently healthy root tissues of the potato plant. In some of their experiments, they were unable to infect uninjured roots with spores under humid conditions because the spores failed to germinate under this environment. When, however, bits of mycelium were placed upon uninjured roots growing under humid conditions, infection quickly followed. Areas of infection were discolored black, and examination of the affected tissues disclosed the presence of the fungus beneath the epidermis. Several investigators (Bewley, 1922; McKay, 1926; McKeen, 1943; and Wilhelm, 1950) independently confirmed the views of the two early workers.

That the fungus can infect the maple tree by avenues of entrance in roots has been assumed by many pathologists without any evidence of actual investigation. Therefore, it seemed desirable to ascertain the facts and incidentally to find out if there is any relation between the soil inoculation methods and the incidence of disease.

Thirty well-established, individually potted, 2- and 3-year-old sugar maple seedlings were employed in 2 series, each with 10 trees for the inoculation and 5 for checks. The inoculum consisted of 4-week-old cultures of the fungus growing on sterilized wheat grains that about half filled 100 cc. bottles. Prior to inoculation of the soil, the jars were vigorously shaken to separate the grains. In the first series the contents of a bottle of inoculum were gently mixed manually with the surface of the soil about each test tree, extreme care being taken to prevent any injury to the surface roots. For the second series, the contents of a bottle were evenly divided and inserted into four evenly spaced holes made by forcing a steel rod to the bottom of the pot. The 2 sets of 5 control pots were inoculated in a comparable manner by using sterile, distilled water containing autoclaved, uninoculated wheat. A 2-inch layer of sphagnum was placed over the surface of the soil of all pots in order to prevent desiccation. An additional precaution to prevent

excessive loss of moisture was to set the pots in an outdoor sunken plot covered with 4 inches of sphagnum. The pots were watered every third or fourth day as necessary in view of the prevailing weather conditions. Proof of pathogenicity was established by laboratory re-isolation of the fungus from tissue transplants taken from infected trees.

It was found (Table 9) that none of the trees in the pots in which the inoculum was applied to the surface of the soil exhibited any outward symptoms of the disease, whereas two of the trees in pots where inoculum was applied deep in the soil showed definite foliar symptoms. About November 15, all of these potted trees were transferred to a fairly well-ventilated root cellar for the winter. In April of the following year, these trees were again set outdoors in the sunken plots. By the end of May the two trees which had displayed disease symptoms the previous year had been killed. One could not attribute the death of these trees to the small wounds made during the process of gathering suspect tissues for cultural diagnosis, since the check trees similarly treated had not been affected. The fungus was isolated from the infested soil in 1952 and again in 1953.

During 1952, three more exposed to deep soil inoculation became infected, whereas only one tree in the surface inoculated pots showed typical wilt symptoms. These three infected trees were positively diagnosed by laboratory pure-culture isolation.

Table 9 — Effect of Method of Soil Inoculation on Number of Trees Becoming Diseased.

Type of Inoculation	No. of Trees Inoculated*	No. of Trees Diseased		Total Diseased
		1951	1952	
Soil Surface	10	0	1	1
Deep soil	10	2	3	5
Control 1	5	0	0	0
Control 2	5	0	0	0

*All trees inoculated June 10, 1951.

The root systems of two diseased trees, one from each series, were exposed by washing the soil away from the roots with a stream of water. Spread of discoloration in the tissues inside the cambium was determined by carefully cutting the wood with a sharp knife. The greenish and sometimes brownish-green discoloration could be traced from the branches down the stem to a small lateral root, below which no discoloration could be detected. This pattern of discoloration strongly suggests that actual invasion by the fungus was initiated through these rootlets. Therefore, the ability of this species of *Verticillium* to infect maple trees by the way of the root system is indicated even though only a limited number of potted trees was involved.

THE PRESENCE AND LONGEVITY OF THE FUNGUS IN THE SOIL

For this study, the potted trees of the preceding section were kept with the

soil intact until 1953. The first attempts to recover the *Verticillium* fungus from the soil utilized the serial dilution method of Brierley, *et al.* (1927), but bacterial and fungus contamination was high. Among the contaminants, a bacterium and an actinomycete exhibited strong antagonistic activity against the *Verticillium* colonies.



Plate 4—Potato tuber not treated with 2% lactic acid prior to the introduction of soil. Note the watery and necrotic tissue resulting primarily from the action of bacteria and yeasts.



Plate 5—Potato tuber treated with 2% solution of lactic acid prior to inoculation with soil. Note the *Verticillium* growing along the walls of the chamber.

Knowledge that potato tubers can be attacked by *Verticillium* (McKay, 1921, 1926) led to the development of a potato tuber technique for the isolation of this fungus from soil. In principle it is similar to the apple technique used by Caroselli (1940) for isolating *Phytophthora cactorum* from soils. The first attempts with potato tubers were not too successful, since numerous rot-producing yeasts and bacteria outdistance growth of this *Verticillium* (Plate 4). The addition of a 2 percent lactic acid solution, however, inhibited these contaminants (Plate 5). A medium sized Irish potato was surface-disinfested with 70 percent alcohol. A flamed cork borer was forced through the tuber, then pulled out containing a lodged potato cylinder. One-half of this cylinder was replaced in its original position in the potato. After the hollow in the tuber was wetted with the lactic acid solution, a small quantity of the soil to be tested was added therein. The remainder of the cyl-

inder, with about $\frac{1}{4}$ inch cut off at one end, was then replaced in the potato, and after the wound surface had been covered with paraffin, the tuber was allowed to incubate at room temperature for 14 to 21 days. The *Verticillium* was usually found growing along the walls of the small chamber in the potato in a reasonably pure state but at least distinguishable and readily isolable. By this technique, it has been shown that the maple wilt fungus can survive in a soil for at least 2 years.

Wilhelm (1950) found that in one case *Verticillium* persisted for 8 years in a soil continuously cropped with non-susceptible hosts. Recently Wilhelm (1955) reported that *Verticillium albo-atrum* is capable of persisting on laboratory media and in soil for 13 and 14 years respectively.

It is known that following the death of an infected plant the fungus may invade the cortical tissues of many plants, herbaceous or woody, and there produce microsclerotia abundantly. These resting bodies are also produced in the vessel elements (Czarnecki, 1923; Rankin, 1914; Rudolph, 1931; Zeller, 1936). The fungus is capable of living saprogenously on dead host roots and saprotrophically on decayed organic matter. All of this material is ultimately incorporated into the soil and can serve as potential inoculum. The relatively abundant occurrence of microsclerotia in plant parts on or near the surface of the soil, their small size and their resistance to drying must, it would seem, make them an important component of the soil dust that can be readily disseminated by air currents.

RELATION OF THE WATER CONTENT OF THE SOIL TO THE DEVELOPMENT OF THE DISEASE

Arborists have long observed that following periods of prolonged drought, Norway and sugar maple trees grown as ornamentals on private grounds, in parks and along streets often display foliar injury resulting from infection by the maple wilt fungus. Several cases of such wilted trees were noted following the drought periods of 1952 and 1953. The effect of soil moisture on *Verticillium* wilt of susceptibles other than maple trees has received considerable attention, but opinions vary.

Rudolph (1931) reviewed the literature on this subject up to 1931. Schneider (1948) showed that guayule plants developed a greater amount of wilt when they were grown in plots which received frequent irrigation, that in field trials this fungus was active in soils in which the moisture levels were above the wilting point, and that maintenance of the upper soil horizons at the wilting point prevented infection. McKeen (1943) reported that when *Verticillium* inoculum was placed in dry soil fallow for approximately 2 months prior to cropping, the fungus invasion of plants was delayed longer than when the pathogen was mixed in wet soil. Baker and Locke (1945) were of the opinion that the epiphytotic of *Verticillium* wilt of annual crops in southern California in 1944 was the result of extended cool, cloudy and humid weather which perhaps maintained a uniform soil moisture. McKay

(1926) reported that *Verticillium* infection of potato was less during periods of drought; he thought that the water in the soil reduced the soil temperature and provided more favorable conditions for the growth of the fungus. Similar observations were made by Zeller (1936) in the case of *Verticillium* wilt of cane fruits, which was found to be severe during periods of continued cold weather.

Ludbrook (1933) in experiments with eggplants growing under greenhouse conditions endeavored to maintain the soil at different percentages of the moisture-holding capacity by adding the required amount of water. He found that the soil moistures between 45 and 95 percent of the soil capacity had little effect upon the incidence of *Verticillium* wilt, except at 84°F. In Isaac's experiments with sainfoin (1946), in which water was added at intervals to bring the soil to various percentages of saturation, it was found that an increase of the soil moisture resulted in a decrease of the incidence of disease.

Bidwell and Childs (1938) and Irish (1937) for infected maple trees, and Haenseler (1922) for peach trees, reported extensive injury after prolonged dry weather in the fall. Strong (1936, 1938) observed that fertilization and heavy soaking of the soil beneath infected maple trees in the early stages of wilt resulted in the disappearance of symptoms and stimulated good growth.

These reports in the literature leave unsettled the question as to what water content of the soil favors the disease once plants are established and are subsequently infected. A set of experiments was designed to procure information along this line.

The trees used were 2-year-old Norway maple seedlings established in soil in 6-inch pots with an inch of washed quartz sand at the base to allow for sub-irrigation and aeration. The soil was sandy loam with a medium amount of available nitrogen, high in available phosphate, low in available potash and with a pH value of 6.1. The pots were paraffin coated on the outside, the bottom hole was plugged with a cork and the soil surface was covered with a 1-inch layer of ground sphagnum. Two glass tubes of half-inch diameter inserted to the bottom on opposite sides of each pot were used for the addition of water to provide and maintain the desired percentage of the water-holding capacity for this particular soil.

The pots were placed on greenhouse benches. Temperature and relative humidity of the air in the greenhouse were not controlled except by daily manipulation of doors, ventilators and steam valves.

Thirty-nine potted trees were divided into four groups, as follows:

- A. Three trees, 2 to be inoculated and 1 as control, with the soil water at 33 and 35 percent of the holding capacity;
- B. Twelve trees, 10 to be inoculated and 2 for checks, with the soil water

39.4 to 40.3 percent and 7 of the inoculated trees with more than 40 percent;

- C. Twelve trees, with the same 10- and 2-relationship as in B with the soil water at 59.3 to 60.6 percent and 7 of these more than 60 percent;
- D. Twelve trees, the same 10- and 2-proportion, with the soil moisture at 79.9 to 81.2 percent and 6 of them more than 80 percent.

Inoculations were accomplished by the spore-suspension method, using strain V-10. The control trees were inoculated with sterile water which was first allowed to come in contact with the surface of sterile, solidified malt agar in petri dishes. All inoculations were made on December 16 and 17. The first symptoms appeared on 5 plants; 2 plants in Group A and 3 in Group B on March 15, after 89 days incubation (Table 10). These seedlings were completely defoliated and dead 165 days after inoculation. The inoculated trees in Groups B, C, and D showed a gradual increase in the number of trees exhibiting wilt up to the end of the experiment (195 days), except that in Group B the maximum percentage (96 percent) was reached after 150 days. The general relationship in all four groups was that the incidence of disease varied inversely with the percentage of the water-holding capacity of the soil.

Table 10 — Relation of Percentage of Soil Moisture to Incidence of Disease in 2-Year-Old Norway Maple Seedlings.

Date 1952	Days after Inocu- lation	Percent of Seedlings Showing Wilt				Checks
		Group A (33%)*	Group B (40%)*	Group C (60%)*	Group D (80%)*	
15 March	89	100	30	0	0	0
21 March	95	100	40	30	10	0
31 March	105	100	60	30	30	0
7 April	112	100	60	40	50	0
21 April	126	100	80	50	50	0
15 May	150	100	90	50	50	0
30 May	165	100**	90	50	50	0
7 June	173	—	90	60	50	0
17 June	183	—	90	70	50	0
22 June	188	—	90	80	60	0
29 June	195	—	90	80	60	0

*Percentage of water-holding capacity of the soil.

**All defoliated and dead.

On June 30 all the trees not exhibiting disease symptoms were placed outside the greenhouse. On August 30, 1 of the 4 trees in Group D unaffected on June 29 was wilted, but none of the others developed disease. Of the 23 trees in Group B, C, and D which developed the disease symptoms in the experiment, 10 were subjected to laboratory culture diagnosis for the presence of the pathogen and examination for discoloration of the vascular tissues. The 13 trees remaining in November were placed in a root cellar where they overwintered. When these trees were

removed the following spring, all leafed out except one in Group D. Then these trees were treated in the same manner as the other 10.

The fungus was isolated from all trees except one each in Groups B and C. Microscopic examination of random sections, from different parts of the discolored tissues from different trees, treated with "cotton-blue" dye by the method described by Van der Meer (1926), revealed the presence of hyphae in most, but not all, of the sections.

The entire xylem area was discolored in two seedlings. In the remaining trees, the discoloration consisted only of a more or less narrow band slightly below and mostly above the point of inoculation. Measurements of the length of the discolored area gave the following figures: Group A, 11.7 inches; Group B, 7.7 inches; Group C, 5.9 inches; Group D, 2.3 inches (extent of discoloration was very limited in 4 of the 6 trees). These data further demonstrate that under the conditions described in these experiments the incidence of disease in young maples is reduced with an increase of the soil moisture.

THE RELATION OF SAPWOOD WATER TO THE INCIDENCE OF DISEASE

Apparent differences between the incidence of *Verticillium* disease in potted 2-year-old maple trees in relation to different amounts of soil water stimulated interest regarding the possible relation of the water content of the sapwood to the degree of wilt and the spread of the fungus within the wood, as indicated by the greenish discoloration of the sapwood.

It is assumed that wood-invading fungi require, in addition to adequate temperature and nutrients, a proper balance of moisture and air in the wood to grow and conduct their normal metabolic activities. Small quantities of moisture may inhibit the growth of these organisms, and conversely, excessive amounts of water may also bring about an inhibition of growth or even death of the organism. Excessive water in the lumina of the cells of the sapwood obviously must reduce the volume of air to the point where the growth of aerobic fungi is no longer possible. Between these limits there will be a favorable range of air-water conditions which not only favors the growth of the fungus but may also stimulate toxin production. The wood moisture contents of individuals of the same species may be affected by such edaphic factors as soil type, soil-moisture variations, vigor of the tree and type of root system. Extensiveness of the root system may also explain the differences of sapwood-moisture content in different trees of the same species growing in the same locality (Table 11).

That there is a relation of the water content of wood to its decay and staining has been demonstrated by several workers. Muench (1909), employing the sapstain organism *Ceratostomella caerulea* Muench and Scots on pine sapwood,

observed that, under conditions of 23 percent of water content in the wet block, staining was slight; that the greatest penetration of the fungus as indicated by the staining of the wood was in the range of 25 to 42 percent wet weight; and that the intensity of staining decreased from this latter point to 59 percent wet weight above which no staining was observed. Muench also obtained similar results with other wood-staining organisms, wood-decaying fungi, and canker-producing fungi.

Snell (1921, 1929) and Snell, *et al.*, (1925) worked for several years on the relation of the moisture content of wood to its decay by five structural timber-destroying fungi and obtained the optimum and complete inhibition points for woods of different specific gravity.

Dick (1936), working with *Sphaeropsis Ellisii* Sacc. and its staining effect on

Table 11—Water Content of Sapwood and Degree of Wilt of Inoculated and Uninoculated Field Trees.

Tree No.*	% Water Content of Undiscolored Sapwood										Degree of Wilt				
	6-7	6-15	6-28	7-16	7-24	8-9	8-23	8-29	9-27	12-4	7-16	7-24	8-9	8-23	8-29
59	57.2	57.6	55.4	57.0	58.5	60.3	57.8	53.3	60.5	63.1	0	0	0	0	1
61	50.3	52.3	52.7	51.9	50.5	48.9	50.8	49.6	53.7	60.3	1	1	1	1	1
62	47.8	47.0	48.4	46.5	43.3	41.5	41.6	47.3	50.7	58.8	3	3	3	3	3
70	52.2	50.2	50.2	48.2	49.4	51.5	53.9	48.9	52.2	56.2	1	2	2	2	2
73	51.9	52.5	51.8	52.2	53.2	50.7	43.3	48.6	53.1	60.9	1	2	2	2	2
74	49.6	50.1	50.8	51.0	50.0	50.0	47.2	48.3	50.6	57.5	0	2	2	2	2
76	48.5	47.9	50.7	45.7	43.1	41.9	43.8	45.8	52.5	55.9	3	3	3	3	3
77	46.9	54.5	54.6	54.8	50.5	51.6	42.9	42.1	51.1	57.1	0	0	0	0	0
80	55.9	54.4	56.1	60.0	55.2	56.2	49.5	53.7	50.4	57.5	0	0	0	0	0
80A	53.2	56.5	52.8	54.3	51.6	53.8	44.6	44.9	49.3	55.7	0	1	3	3	3
83	56.5	58.9	59.8	57.8	57.7	55.9	55.9	54.2	46.0	59.0	1	1	1	1	1
84	56.1	55.4	53.1	54.1	56.1	54.8	57.1	50.9	46.2	58.3	0	0	0	0	0
240	58.1	58.3	54.6	57.0	58.1	58.2	51.5	57.2	56.3	62.9	0	1	1	1	1
244	54.5	55.2	50.3	49.8	49.7	52.0	46.9	45.9	51.6	61.4	1	1	1	1	1
253	41.9	50.1	49.4	47.4	46.9	47.5	46.5	45.0	44.6	54.3	2	3	3	3	3
254	50.9	55.9	58.9	58.7	52.9	50.0	51.4	55.2	53.8	59.9	0	0	0	0	0
264	48.0	48.9	47.6	42.5	41.9	45.4	40.1	42.0	45.0	62.0	2	2	3	3	3
265	50.2	47.7	50.0	47.3	49.6	44.6	48.9	47.7	48.1	61.1	2	2	2	2	2
270	55.5	57.2	56.9	57.5	55.5	55.4	46.4	52.3	53.9	58.8	0	0	1	1	1
272	48.8	56.9	54.4	52.9	51.7	45.6	43.4	42.8	46.7	59.2	1	2	2	2	2
277	42.8	47.1	47.0	40.9	41.1	41.7	41.5	40.0	42.3	58.1	3	3	3	3	3
279	42.2	45.2	46.3	41.6	42.2	42.7	40.1	40.0	43.4	63.2	2	3	3	3	3
337	40.8	60.7	46.7	47.1	41.8	40.0	54.9	44.8	47.5	59.9	2	2	2	2	2
339	50.3	54.2	51.1	52.4	49.2	48.1	40.7	39.0	45.2	62.7	0	2	2	3	3
340	47.9	51.6	54.0	50.4	54.6	48.9	44.5	45.9	49.8	60.3	1	2	2	2	2
C-1	51.1	61.6	53.9	56.7	58.9	53.2	58.8	52.4	56.2	64.9	1	1	1	1	1
C-4	48.7	54.4	54.9	55.6	54.1	50.0	53.1	51.8	48.1	57.6	1	1	1	1	1
C-12	53.5	58.0	59.4	53.7	53.6	54.2	47.6	50.4	52.7	62.6	1	1	1	1	1
C-15	58.2	60.2	57.6	58.1	49.4	49.2	54.5	49.0	53.8	58.4	0	0	0	0	0
68B	41.3	41.3	56.8	50.2	45.7	42.9	55.8	41.1	49.4	56.1	0	0	0	0	0
82	56.8	56.8	46.2	52.2	51.8	51.2	51.8	51.1	48.9	56.0	0	0	0	0	0
89A	54.6	59.9	54.9	50.3	51.4	45.8	45.1	54.5	62.0	61.2	0	0	0	0	0
335	57.2	53.2	49.4	57.8	44.5	46.1	43.9	42.4	44.1	57.8	0	0	0	0	0
338	42.5	50.8	42.2	45.3	49.5	48.6	46.9	46.8	44.5	57.5	0	0	0	0	0
C-7	54.1	52.8	55.0	49.1	48.5	45.3	46.5	57.5	52.3	59.0	0	0	0	0	0
C-10	47.1	57.7	54.1	52.9	44.9	48.9	43.7	48.6	51.9	61.5	0	0	0	0	0

*The 29 trees to the heavy black line were inoculated with *Verticillium* V-10 on June 7, 1950; the 7 trees below the heavy black line were the uninoculated controls.

the sapwood of white pine, found that very slight staining took place in inoculated sapwood in the winter green condition and that the degree of staining increased as the moisture of the sapwood decreased to 60 percent of the winter green condition below which staining decreased.

As has been shown in the preceding section of this paper, opinions are varied regarding the relationship of water content of the soil to the incidence of the *Fer-ticillium* wilt disease but most writers have regarded drought as distinctly favorable to the disease. A search of the literature, however, failed to disclose a logical explanation for this phenomenon. In general, discussions dismiss the matter with ambiguous references to a decrease of host vitality resulting from prolonged periods of drought and in no instance was there any mention or speculation concerning the moisture-air relationship of the wood to the development of disease.

In an endeavor to obtain some information on this subject, observations and experiments involving well established, apparently healthy, and actively growing trees were carried out during the summer and fall of 1951. The purpose of the study was fourfold: (1) to ascertain the relation between the air-water balance in the wood and the severity of the wilt symptom; (2) to compare sapwood water content with the extent of vascular discoloration occurring during the season; (3) to compare the water content of discolored and undiscolored wood; and (4) to find out if there was any correlation between the water content of the soil around the test trees and the water content of the sapwood.

Thirty-six trees, varying in size from $1\frac{1}{2}$ to 5 inches DBH and in height from 15 to 35 feet, were selected at random in a 2-acre plot whose soil comprised about 2 feet of a fine sandy loam soil over gravel. This soil was found to be low in nitrate nitrogen, ammonia nitrogen, phosphorous and manganese, medium in magnesium and aluminum content, and to have a pH value of 5.67. Organic matter, of the sort commonly present under woodlot conditions, (primarily decomposed leaves and decaying woody parts), was medium in quantity.

Water content of the sapwood is expressed as percentage of the oven-dry weight — the constant weight obtained in standard practice by drying at 100°C . for 48 hours. It should be noted that in these determinations "moisture" or water includes any other substance present in the green wood which may be volatile at 100°C .

Samples used in this study were taken, wherever possible, from the same side of the tree and in an area above the point of inoculation. In order to minimize structural weakening of small trees, the samples were procured from scattered areas of the trunk. This procedure was considered to have little effect on the results, since it was demonstrated by Newlin (1917) that hardwoods exhibit a fairly uniform distribution of water in the same tissues throughout the tree.

The test specimens were obtained by making a triangular cut into the trunk of the tree with a half-inch chisel. Only clear wood without knots or abnormalities was used, whether the samples were from inoculated or uninoculated trees or from discolored or undiscolored tissue, in order to eliminate any possible influence of structural defects upon the calculations. Immediately after cutting, the samples were carefully wrapped in filter paper and then in waxed paper, both of which had been oven-dried and weighed. Each wrapped sample was then taken to the laboratory and weighed, the waxed paper discarded, and the wood samples in the filter paper were oven-dried for the final determination of the moisture content.

Of the 36 trees selected for this study, 29 were marked for inoculation with *Fusicillium* and 7 for controls. Inoculation was accomplished by introducing 5 ml. of a concentrated spore suspension into the trunk. The control trees received 5 ml. of water which had been allowed to come in contact with the surface of sterile nutrient agar. These inoculations were made on June 7, 1951.

For the determination of the moisture content of the soil, samples were taken with a soil auger to a depth of 2 feet from around each tree on the same dates as those upon which the wood specimens were gathered. Five or six scattered borings were combined and mixed thoroughly, and from this mixture a 10-gram sample was oven-dried for the calculation of water content. It was considered that this spacing and the depth of the borings was adequate for the purpose because of the regularity and shallowness of rooting of the trees.

Upon completion of the study in December 1951, 14 of the 29 inoculated trees were cut down and dissected for a determination of the spread of the fungus as indicated by the greenish or blue-green discoloration of the sapwood above and below the point of inoculation (see last column of Table 12). At this time, aseptic tissue plantings for re-isolation of the fungus from the discolored wood were made to malt agar. These were positive in all cases. The results of the observations and the experimentation are recorded in Table 11.

Of the 29 trees inoculated on June 7, 1951, 24 showed wilt expressed in various degrees by August 29. The fungus was isolated from all of these trees and also from 2 more (numbers 77 and C15) which developed no wilt within 6 months.

The average percentages of the water contents of the sapwood of inoculated and uninoculated trees are compared graphically in Figure 3. With the exception of the averages of July 24 and August 9 showing 2.5 and 3 percent differences, respectively, between these two groups, there is reasonably close agreement. The disparities may possibly be attributable to an insufficient number of uninoculated check trees, although a variety of explanations is conceivable. Considerable variation in water transmission through sections of healthy elm trees was found by Zentmyer, *et al.* (1946). In any event, the differences thereafter were only 0.5 percent until September 9 when the averages became practically the same and continued so until the end of December 4.

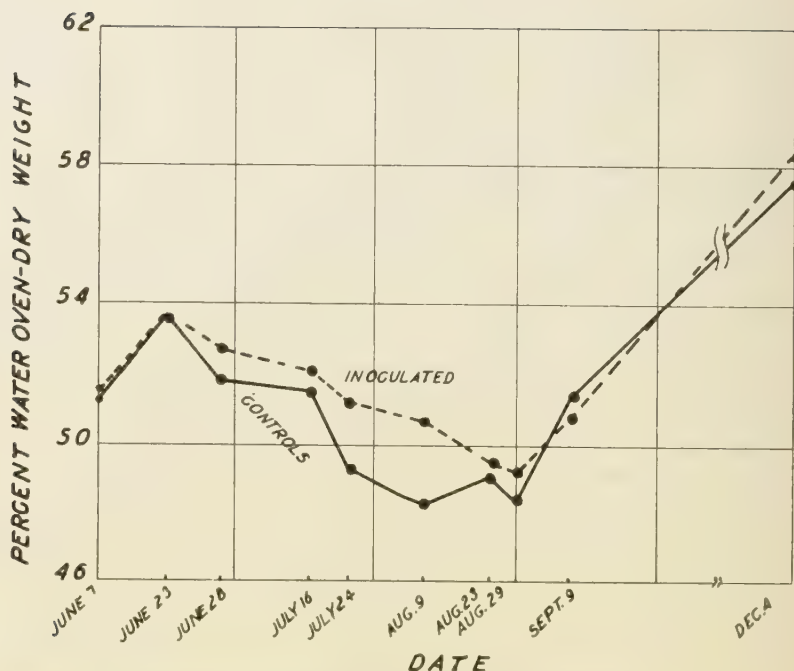


Figure 3—Average water-content (%) of inoculated and check trees.

With regard to the relation of water content and the degree of wilt, there appears to be a general tendency toward an increase in the wilt symptoms with a decrease in the water content of the sapwood and vice versa. A consideration of a few individual examples are enlightening:

Tree 62 — 3-degree of wilt from the beginning, with the water content from 46 to 48 percent, then 43 percent, 41 percent, and finally 47 percent.

Tree 76 — about the same as tree 62, with a few percentages a little higher.

Tree 277 — all water-content measurements between 40 and 43 percent after July 1; 3-degree wilt throughout.

Tree 339 — no wilt at 50 to 54 percent, 2-degree wilt when the water content dropped to 49 and 48 percent, and 3-degree wilt when the water content dropped to 39 to 40 percent.

Tree 264 — when the percentage of water was above 45, 2-degree wilt prevailed but when water content dropped below 45 to 40 percent, the wilt was 3-degree.

Tree 80A — with the water content at 52 to 56 percent, no wilt; when the water content dropped to 51 percent, 1-degree wilt; and with a further lowering to 44 percent, 3-degree occurred.

Tree 59 — with the water content 57 to 60 percent, no wilt; with a drop to 53 percent, 1-degree wilt.

Tree 83 — with the water-content 56 to 59 percent, no wilt; but when it dropped to 55 and 54 percent, 1-degree developed.

Trees 240, 244, 270, C1, C4, and C12 — with the water contents 45 to 54 percent, 1-degree wilt only took place.

The results from water content analysis of all the trees are just as striking. Figure 4 shows the frequencies of the different wilt classifications recorded from the first appearance of wilt symptoms on July 6 and continued to August 29, after which date wilt could not be accurately discerned because of incipient yellowing of foliage in September. The main points appear to be as follows:

No wilt occurred at a sapwood water content higher than 59 percent, and there were only three cases above 57 percent.

One-degree wilt occurred from roughly 59 down to 45 percent water

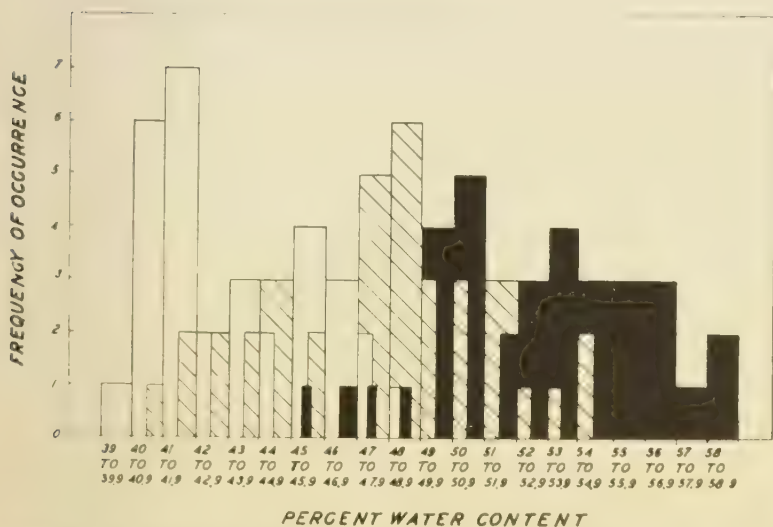


Figure 4—A comparative histogram showing the relation between the amount of sapwood water and the degree of wilt of inoculated field-grown trees. The degrees of wilt — 3, 2, & 1 — are represented by plain, diagonal, and solid bars respectively.

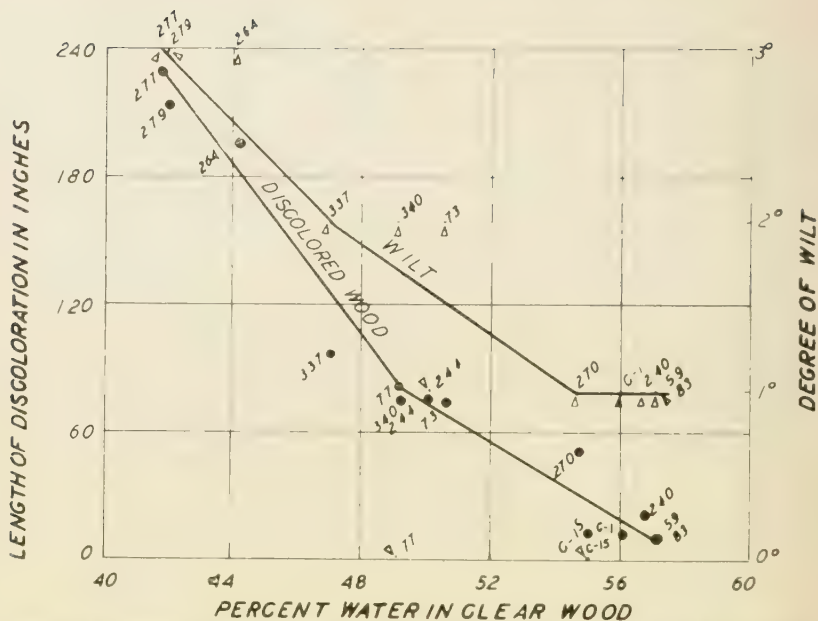


Figure 5—Smooth curve showing the extent of green-discolored wood and degree of wilt in relation to the mean water-content of clear sapwood of field-grown trees.

content with the peak at 50 to 51 percent.

Two-degree wilt developed at water content of 55 to 40 percent, with the peak about 48 percent.

Three-degree wilt resulted between 48 and 39 percent with a peak at 41 percent.

The indications are that wilt symptoms appear only as the water content of the sapwood is lowered from the saturated or winter-green condition, and that within limits, as the water-content decreases, the amount and extensiveness of the wilt increases (Figure 5).

The relation of the green streaking or discoloration accompanying the progress of the fungus mycelium in the sapwood to the water content of the sapwood of 14 trees is represented in Figure 5, based on the last pair of columns in Table 12. Despite the scattering of the points on the graph, there is a strong indication that the extent of discoloration increases with a decrease in water content of the sapwood and in a reasonably regular manner.

A comparison of the average water content of discolored and undiscolored

wood was made from trees 73, 244, 253, and 340 for the period from July 24 to September 27 (figures in the next to last pair of columns in Table 12). These four trees were selected because they provided a large number of discolored samples. The average water contents, in order, for the trees numbered above for the discolored and undiscolored wood were (in percentages): 49.77 vs. 45.84, 49.21 vs. 44.76, 46.08 vs. 41.92, and 48.71 vs. 44.96. An average difference of about 4.12 percent is evident. It is possible that this consistent difference was due to a partial plugging of the vessels by the mycelium and a consequent interference with the normal movement of water through the invaded xylem elements.

Table 12 — Water Content and Extent of Undiscolored and Discolored Sapwood in Inoculated Field Trees.

Tree No.	Percent of Sapwood Content		Green Discoloration in Inches	
	July 24 Undiscolored	September 27 Discolored	From Point of Inoculation Downward	From Point of Inoculation Upward
59			10	2
73	49.77	45.84	72	.75
77			81	1
83			11	2
240			26	2
244	49.21	44.76	76	1.5
264			196	1.5
270			51	1
277			228	2.5
279			218	2
253	46.08	41.92	—	—
264			196	1.5
270			51	1
277			228	2.5
279			218	2
337			95	1
340	48.71	44.96	76	1.5
C-1			15	1
C-12			24	1

No positive indication resulted from a considerable volume of figures obtained in one study of the relation of the water content of the soil to that of the sapwood. In a number of instances there seemed to be a correlation (i.e., as the water content of the soil varied, so did the water content of the sapwood). On the other hand, in about as many cases the reverse was true. It appears that a much more complicated study involving more frequent sampling and also certain meteorological information would be necessary before any satisfactory conclusions are possible. Consequently, the detailed data from the observations are not presented here.

Influence of Tree Condition on Pathogenicity of the Fungus

From the earliest observations made on the maple wilt disease, it has been assumed that the condition of the tree played an important role in the incidence of disease. Accordingly, arborists in their efforts to combat this disease have fos-

tered practices which would promote tree vigor. To gain evidence on this subject, three different experiments were performed.

EFFECT OF DEFOLIATION

Fifteen small field trees approximately 4 to 6 feet high were stripped of leaves on June 10, 1952. Ten of these were inoculated with a spore suspension of *Verticillium* V-10 on June 16, 1952, and the 5 remaining uninoculated trees were used as checks to determine the effect of stripping. Also, five additional similar, but unstripped, trees were inoculated as a further check.

The trees which were defoliated produced a new crop of leaves by August 10, 1952. During this period, no wilt or dieback of the branches was observed on any of the trees stripped prior to inoculation. On the other hand, the unstripped, inoculated trees exhibited an average wilt condition of 42 percent. Stripped, uninoculated trees showed no wilt or dieback of the twigs.

On May 7, 1953, tissue samples were taken from all trees inoculated in 1952. The fungus was isolated from all inoculated, unstripped trees and from 8 of the 10 inoculated trees which were stripped of leaves. In the latter, the greenish discoloration was confined to a limited area near the point of inoculation, whereas in the former group the discoloration extended upward for some distance. Defoliation appears not only to suppress the incidence of disease, but to check its extent.

EFFECT OF ROOT INJURY

Roots of 15 sugar maple trees 1 to 4¼ inches DBH were injured by vigorously shaking the trunk and cutting with a spade. Ten trees were inoculated with a spore suspension of V-10 and 5 were reserved for checks. Ten trees with uninjured root systems, of approximately the same size as above, were also inoculated. Resulting data recorded in Table 13 show a greater expression of wilt on trees with injured root systems.

Table 13 — Effect of Root Injury on the Incidence of Disease Resulting from Inoculation with *Verticillium*.

No. of Trees	Date of Root Injury	Date of Inoculation	Average Wilt* 8-31-52
10	6-13-52	6-23-52	2.1
10		6-23-52	1.1
5	6-13-52		0.0

*Expressed as index 0-3.

EFFECT OF VARIOUS SOIL AMENDMENTS ON MAPLE WILT

Since *Verticillium* is fundamentally a soil organism, long known to infect a vast number of unrelated hosts in the temperate regions of the world, control meas-

ures other than the utilization of resistant plants have been directed to the use of various soil fumigants and miscellaneous soil amendments. The application of such measures as removal of 3 feet of soil from affected areas, suggested by Keyworth (1942), drenching of the soil with mercuric chloride reported by Rudolph (1931), soil fumigation with chloropicrin or carbon disulphide and ethylene dibromide found to be effective by Young (1940) and Smith (1940), respectively, is not practical for the control of the disease in well established trees; the soil could be disinfested, but the trees would be killed. Thus, soil amendments appear to be a feasible approach.

Experiments in this direction were undertaken by Gallegly (1949), who noted more pronounced symptoms in tomato plants when soil nitrogen, potash and phosphates were increased. Wilhelm (1951) noted that certain amendments high in nitrogen, such as blood meal, fish meal, and ammonium sulphate added to *Fusicillium*-infested soils, reduced the infective powers of the fungus. Also, Clark (1942) and Leach (1942) found that nitrogenous fertilizers reduced diseases caused by various fungi.

Information on the control of maple wilt by the use of soil-conditioning agents is scant. Occasional references are available regarding the beneficial effects of watering and "feeding" infected trees (Felt & Rankin, 1932; Gravatt, 1926; Irish, 1937; Rudolph, 1931; Strong, 1936, 1938). The first actual experimentation was carried out by Irish (1937), who reported that the addition of 6 pounds of aluminum sulphate to 20 pounds of a 10-6-3 fertilizer markedly reduced *Fusicillium* wilt symptoms of three American elm trees. The fertilizer was applied to the soil in May and the trees were pruned and watered periodically during the growing season. No wilt was noted for the remainder of the year.

Experiments were conducted in 1951 and 1952 aimed at solving two questions: the host response to the availability of organic and inorganic nitrogen and the amount of material that would be beneficial.

Sugar maple trees from 13 $\frac{1}{4}$ to 6 inches DBH were divided into randomized groups and were separately treated with ammonium sulphate, sodium nitrate, Nuregon, Milorganite, cyanamide, and a 6-8-7 "tree food". In the individual treatments these compounds were applied, at the rate of $\frac{1}{8}$, $\frac{1}{4}$, and $\frac{1}{2}$ pounds of nitrogen for every inch DBH for each tree. Materials were added to the soil in one series 1 month before inoculation and in the second series 9 months before. These amendments were broadcast on areas marked off for each group and then worked into the soil by light cultivation with a rake. Since in some instances the amount of material was small for the area to be covered, the volume was increased by the addition of an inert filler. In order to eliminate possible competition for fertilizer elements, the few herbaceous plants growing in the shade of these trees were weeded out.

The soil was a well drained sandy loam. Soil samples collected from each plot were tested for pH with an all-electric Beckman meter before and after treatment. Inoculation was by the spore-suspension method. In addition to inoculated check trees without soil amendments, uninoculated trees were treated with similar amounts of chemicals in order to check for phytotoxicity.

Since it had been found in inoculation studies that small trees exhibited unusually severe wilt symptoms, 30 trees ranging in size from $\frac{3}{4}$ to $\frac{7}{8}$ inch DBH and approximately 8 feet high were used for the purpose of comparing the effects of sodium nitrate and ammonium sulphate upon the degree of wilt. It was believed that the use of these smaller trees might give a more accurate representation of the percentage of wilt symptoms for the entire tree. In order to prevent the overlapping of treatments, the trees were carefully grouped and the area to be treated was marked off with string. Ten trees were treated with sodium nitrate and 10 with ammonium sulphate, both at the rate of $\frac{1}{4}$ pound of available nitrogen for each inch DBH. Control trees were divided into two groups; one consisting of 8 trees which were not inoculated, the other of 2 trees each differently treated with equivalent quantities of ammonium sulphate and sodium nitrate. The latter treatment was undertaken in order to measure any possible effects of the chemicals upon the roots or the foliage.

Soil applications were made on September 7, 1951, and repeated on April 7, 1952, prior to inoculation on May 13, 1952. Since the area where these trees were growing was flooded in early spring and signs of soil erosion were noted, the second application of one-half the amount in the first one was made for the purpose of compensating for any leaching of the chemicals which may have occurred. Herbaceous plants, which were growing luxuriantly in this plot, were removed prior to the addition of the chemicals. These elements were applied and mixed into the soil in the manner previously described for large trees. This plot was low and drained by a brook located about 60 feet north of the nearest tree. The average pH from several soil tests was found to be 4.5. Inoculation of the trunks was made by the agar-disc method (Plate 5).

Records of wilting were made at the outset and intervals throughout the season. Degree of wilt was scored as 1, 2, or 3 for the large trees, and expressed percentage-wise for the small trees.

The addition of certain fertilizers to the soil around trees 1 month and 9 months before inoculation brought about a reduction of the disease in some cases and in others gave no evidence of control (Tables 14 & 15). The results were more or less parallel in both small and large trees.

As shown in Tables 14 and 15, all the amendments with the exception of sodium nitrate reduced the incidence of disease in the large trees. In general, a more beneficial response was observed when one-half pound of each chemical

except sodium nitrate) was applied for each inch DBH. With the 6-8-7 "tree food", however, best results were noted with $\frac{1}{8}$ and $\frac{1}{4}$ pound of available nitrogen. Compared with the degree of wilt observed on the check trees, addition of organic compounds such as Nugreen and Milorganite in all amounts produced only slight control. Response to cyanamide was somewhat effective in reducing the wilt.

The best control was obtained in the plots treated with ammonium sulphate. This was indicated by an average score for all treatments of 1.6, compared with the controls with a wilt score of 2.6. In these plots control of the disease was consistent from the onset of the symptoms to the end of the 1951 season. The recording made at the end of the 1952 season also showed good inhibition of the disease as compared with the amount of wilt on the check trees and on those trees given the other treatments.

The most severe wilt symptoms, even more pronounced than those exhibited by the check trees, occurred on those treated with sodium nitrate. The larger the quantity of this chemical applied (to the maximum of $\frac{1}{2}$ pound), the more extensive were the wilt symptoms.

A single recording of the results of the application of soil amendments to large trees 1 month prior to inoculation was made in 1952 (Table 14, last column). These data show a decrease of wilt symptoms from the condition in 1951 in every case, even with sodium nitrate. It is worth pointing out, however, that the decrease

Table 14 — Effect of Soil Amendments Applied May 11, One Month Prior to Inoculation June 11, upon the Incidence of Verticillium Wilt in Large Trees.*

No. of Trees Treated	Treatment	Lbs. N, per Inch DBH	Soil pH		Mean Wilt					
			Before Treat- ment	After Treat- ment	7-16-51	7-24-51	8-9-51	8-23-51	8-29-51	8-30-52
10	None	—	5.62	5.60	1.1	1.4	1.9	2.4	2.6	1.9
9	NuGreen	$\frac{1}{8}$	5.60	5.62	1.7	1.7	2.2	2.3	2.5	1.8
8	NuGreen	$\frac{1}{4}$	5.63	5.63	1.0	1.7	2.1	2.4	2.5	1.7
6	NuGreen	$\frac{1}{2}$	5.60	5.61	1.1	1.2	1.7	2.0	2.3	1.5
9	$(\text{NH}_4)_2\text{SO}_4$	$\frac{1}{8}$	5.60	4.54	0.3	0.4	0.9	1.2	1.7	0.7
9	$(\text{NH}_4)_2\text{SO}_4$	$\frac{1}{4}$	5.47	4.40	0.0	0.2	0.5	1.0	1.6	0.9
8	$(\text{NH}_4)_2\text{SO}_4$	$\frac{1}{2}$	5.55	4.60	0.0	0.1	1.0	1.4	1.5	0.9
9	NaNO_3	$\frac{1}{8}$	5.59	5.62	1.2	2.1	2.1	2.5	2.7	2.0
9	NaNO_3	$\frac{1}{4}$	5.59	5.81	1.0	1.9	1.9	2.3	2.7	2.0
7	NaNO_3	$\frac{1}{2}$	5.60	5.69	1.3	2.1	2.4	2.7	2.8	2.7
5	6-8-7	$\frac{1}{8}$	5.60	5.61	1.0	1.1	1.6	1.6	2.0	1.6
5	6-8-7	$\frac{1}{4}$	5.60	5.60	1.0	1.1	1.4	1.4	2.2	1.7
5	6-8-7	$\frac{1}{2}$	5.60	5.69	1.0	1.4	1.4	2.0	2.6	1.8
8	Milorganite	$\frac{1}{8}$	5.64	5.63	0.9	1.2	1.3	2.0	2.4	1.5
8	Milorganite	$\frac{1}{4}$	5.62	5.62	1.2	1.7	1.8	2.1	2.4	1.5
6	Milorganite	$\frac{1}{2}$	5.57	5.61	0.5	0.5	1.4	1.6	2.0	1.3
5	Cyanamide	$\frac{1}{8}$	5.61	5.60	1.0	1.0	1.4	1.4	2.0	1.2
5	Cyanamide	$\frac{1}{4}$	5.62	5.62	1.0	1.0	1.6	1.6	2.1	1.6
7	Cyanamide	$\frac{1}{2}$	5.60	5.60	0.9	1.0	1.9	2.3	2.3	1.8

*Uninoculated controls for each chemical exhibited no injury.

Table 15 — Effect of Soil Amendments Applied 15 August 1951 Prior to Inoculation May 12, 1952 on Incidence of Verticillium Wilt in Large Trees*.

No. of Trees Treated	Treatment	Lbs. N ₂ per In. DBH	Soil pH		Mean Wilt—1952					
			Before Treatment	After Treatment	5 July	22 July	6 Aug.	20 Aug.	31 Aug.	Aug.
11	None		5.60	5.61	1.0	1.6	2.0	2.4	2.5	
5	NuGreen	1/8	5.53	5.50	0.2	1.6	1.6	1.6	2.0	
5	NuGreen	1/4	5.57	5.51	0.8	1.8	2.0	2.0	2.2	
5	NuGreen	1/2	5.61	5.53	1.2	1.6	2.0	2.2	2.3	
5	(NH ₄) ₂ SO ₄	1/8	5.62	4.31	0.4	0.4	1.0	1.0	1.3	
5	(NH ₄) ₂ SO ₄	1/4	5.61	4.40	0.4	0.4	0.8	1.2	1.8	
5	(NH ₄) ₂ SO ₄	1/2	5.57	4.43	0.2	0.2	0.4	0.8	1.2	
5	NaNO ₃	1/8	5.54	5.74	1.4	1.8	2.0	2.8	2.8	
5	NaNO ₃	1/4	5.72	5.83	1.4	1.8	2.0	2.2	2.6	
5	NaNO ₃	1/2	5.60	5.69	0.5	1.0	2.0	2.2	2.4	
3	6-8-7	1/8	5.60	5.63	1.0	1.0	1.0	2.0	2.0	
2	6-8-7	1/4	5.66	5.69	0	1.5	2.0	2.0	2.5	
5	6-8-7	1/2	5.49	5.51	1.2	1.8	2.0	2.4	2.5	
3	Milorganite	1/8	5.70	5.61	0.3	1.3	2.0	2.0	2.5	
3	Milorganite	1/4	5.62	5.60	0.3	1.0	1.7	2.0	2.0	
3	Milorganite	1/2	5.57	5.64	1.0	1.1	2.0	2.3	2.3	
5	Cyanamide	1/8	5.63	5.59	0.6	1.4	1.8	2.0	2.2	
5	Cyanamide	1/4	5.50	5.60	0.6	1.4	1.8	2.0	2.2	
5	Cyanamide	1/2	5.55	5.63	1.0	1.4	1.6	1.6	1.8	

*Uninoculated control trees for each chemical showed no wilt or injury.

was greater with the ammonium sulphate than with the sodium nitrate.

Although wilting was less in the great majority of comparable observations, a similar pattern of reduction of wilt is to be noted for the large trees to which the soil amendments were applied 9 months before inoculation as compared with the applications made 1 month before (Table 15 and 14, respectively).

With respect to both applications, slight injury to a few leaves of the lower branches was apparent on uninoculated trees treated with 1/2 pound of ammonium sulphate. All other rates of application of this chemical, as well as all the other fertilizers, did not cause any leaf abnormalities. Several superficial rootlets were examined but no injury that could be associated with the amendments tested was detectable.

Small trees treated with sodium nitrate and ammonium sulphate prior to inoculation displayed a progress of the wilt similar to that observed on larger trees. Wilt symptoms were, however, much more striking on these than on the larger trees. The effectiveness of these two soil treatments is shown graphically in Figure 6. Compared with the inoculated check trees, the symptoms of disease were more pronounced in the sodium nitrate plot and markedly less on the trees treated with ammonium sulphate. On August 29, 1952, the mean percentage of 10 trees treated with sodium nitrate was 39 percent, whereas that of the check trees was 35 percent—a difference of 4 percent more wilt for the treated trees. In contrast to this situation, the mean wilt of trees treated with ammonium sulphate was 20 percent.

In the spring of 1953, 5 of the nitrate-treated trees and 4 of the check group

were dead. Although all of the trees treated with ammonium sulphate were alive during the ensuing growing season two showed some wilt and had dull-looking leaves; they were slightly blanchd.

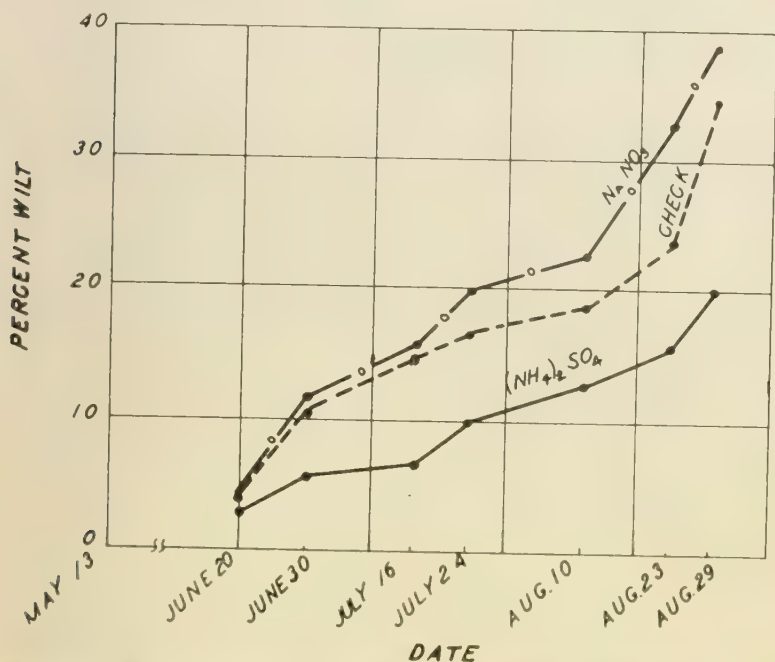


Figure 6—Effect of fertilizers on the incidence of disease in young sugar maple trees.

Soil amendments high in nitrogen vary in their ability to reduce the incidence of maple wilt caused by *Fusicillium* sp. The source of available nitrogen, whether organic or inorganic, does not appear to be the factor responsible for disease control. Better control was afforded by ammonium sulfate than by sodium nitrate, which, as noted, stimulated wilt symptoms. All organic materials gave some degree of control but cyanamide produced the best results; at least it appeared to have more lasting effects than the other organic materials.

It was reported by Harnseler (1926) and Guba (1934) that *Fusicillium* wilt of eggplant is less severe in acid soils. That the reduction of wilt in maple trees, under the conditions of these experiments, may be brought about by a lowering of the soil pH after the addition of ammonium sulphate does not seem valid. There

was some change to a lower pH around some of the larger trees but no appreciable difference in pH occurred in the area where the small trees were growing. Severity and control of the disease were about the same in these areas.

The use of ammonium sulphate was found by Wilhelm (1951) to reduce the inoculum potential of *V. albo-atrum* in soils. In the present experiments, the fungus was introduced directly into the tree, thereby eliminating this factor.

It appears unlikely that the salutary effects of the ammonium sulphate were a result of the rapid availability of nitrogen which brought about an improvement in the resistance of the host. The nitrogen in the sodium nitrate was also quickly available. Donandt (1932) reported that higher nitrogen favored the disease in potatoes. Streets (1934) found that heavy applications of ammonium sulphate reduced the disease of pecans caused by *Phymatotrichum omnivorum* (Shear) Duggar.

Whether or not there is any chemotherapeutic reaction occurring when ammonium sulphate dissociates into different ions in the soil is a possibility which has not been studied. Also, the therapeutic value of ammonium sulphate *per se* has not been tested to date.

The results at hand throw some light on the possible practical control of maple wilt by the addition of certain beneficial chemicals to the "tree foods" used commercially. This practice appears, however, to be limited to ornamental trees having an extensive and accessible root system.

NUTRITION OF VERTICILLIUM V-10 AND PRODUCTION OF TOXIN

The initial phase of the study of the toxin and the problems involved was a determination of the nutritional conditions necessary for the production of maximum amounts of toxic metabolite by the fungus. Obviously, it was desirable first to be able to produce the optimum growth of the fungus.

Nutrition of the Fungus LIQUID MEDIA

It was found (Table 16) that the fungus grows well on all the liquid synthetic media tried except Leonian's and this latter medium was improved by increasing the peptone from 5 to 10 grams per liter.

The best media was found to be a modification of Richard's medium ("Richards-C" in Table 16), the original Tochinai and a modification of it (Tochinai-C), and a formulation "H". The Tochinai medium was found to provide a some-

Table 16—The Effect of Various Nutrients* on the Growth of Verticillium V-10 in Liquid Media.

Media**	MgSO ₄ ·7H ₂ O	KH ₂ PO ₄	KHPO ₄	KCl	Yeast Extr.	Caso-Amino Acid	FeCl ₃	FeSO ₄	KNO ₃	NaNO ₃	NH ₄ NO ₃	(NH ₄) ₂ PO ₄	Ferric Tartrate 10%	Peptone	Bactotryptose	Sucrose	Dextrose	Maltose	Malt Extr.	Succinic Acid	Asparagine	Growth of the Fungus†
A-1	1	1.5			2	2	.01										25				1	++
A-2	1	1.5			1	1	.01										25				1	++
B	1	1.5			2	2	.01								2		15				1	++
C	0.5	1.5			2	3							1ml				15				1	++
D	0.5	1.5			1	2							1ml				20				1	++
E	1	1.5			2	2	.01										10				1	++
F	0.5	1.5								.5				1			20				1	++
G	1	1.5			2		.01										20				2	++
H	2.5	5.0					.01										30				2	++
Richards	2.5	5.0					.01					1									2	++
Richards-C	0.5	5.0					.01									50					2	++
Czapek	0.5	1.0						.01								25					1	++
Czapek-C1	0.5	1.0		.5				.01								30					1	++
Czapek-C2	0.5	1.0		.5				.01				1				30					1	++
Czapek-C3	0.5	1.0						.01	1.5							30					1	++
Tochinai	0.5	1.5												10.0							2	++
Tochinai-C	0.5	1.5												0.6							2	++
Leonian	0.6		1.2											10.0							2	++
Leonian-C	0.6													10.0							2	++

*Nutrients are expressed as grams in 1000 ml. of distilled water, except where otherwise noted.

**The letters A to H refer to various formulations tried; Richards-C refers to our modification of Richards solution, etc. †Growth is represented as fair (+), good (++), and excellent (+++).

what better production of spores than the others, and therefore this was used in all cases in which spores were important for any purpose.

A luxuriant formation of mycelial mat was also produced in a decoction made by boiling young maple twigs for 20 minutes. A decoction of older twigs was not satisfactory.

There appears to be some relation between the pH of the medium and the amount of growth. The following weights of mycelial mats represent 21 days' growth of the fungus maintained at 22°C. in 250 ml. Erlenmeyer flasks containing modified Richards' medium buffered with citric acid (Gortner, 1938) :

pH	3.2	3.5	4.0	4.5	5.0	5.5	6.3	6.8
mg. wt.	10	17	27	114	115	210	210	104

SOLID MEDIA

Experiments were conducted for the purpose of determining the response of *Verticillium* V-10 to different sources of carbon and nitrogen. Petri dishes containing Czapek's agar medium about 3 mm. thick were inoculated with 3 mm. discs of the culture, with five replicates to a test. The sources of carbon were 1, 3, 5, and 10 percent each of dextrose, glycerine, maltose, and sucrose. The sources of nitrogen were casein, 1-asparagine, gelatin, peptone and sodium nitrate in amounts of 0.1, 0.5, and 1.0 percent. Results were taken in terms of average diameter of colony growth.

The records in Table 17 and Figure 7 show that the fungus grew well on media containing maltose and sucrose at the 3 and 5 percent and glycerine at the 1 percent levels. In all cases, there was a marked reduction in the size of the

Table 17 — Effect of Various Amounts of Different Nitrogen and Carbohydrate Source upon the Growth of *Verticillium* V-10 on Nutrient-Agar Media.

Nitrogen Source			Carbon Source		
Material	% in 1000 ml.	Avg. Diam. of Colonies in mm.	Material	% in 1000 ml.	Avg. Diam. of Colonies in mm.
Asparagine	0.1	66.3	Maltose	1.0	60.0
	0.5	67.0		3.0	71.0
	1.0	70.0		5.0	79.2
NH ₄ NO ₃	0.1	74.0	Dextrose	10.0	66.0
	0.5	75.0		1.0	28.0
	1.0	79.0		3.0	35.7
NaNO ₃	0.1	74.0	Sucrose	5.0	53.0
	0.5	74.0		10.0	42.0
	1.0	77.3		1.0	37.0
Casein	0.1	74.0	Glycerine	3.0	67.1
	0.5	73.0		5.0	74.8
	1.0	64.0		10.0	49.0
(NH ₄) ₂ SO ₄	0.1	64.3		1.0	63.0
	0.5	57.2		3.0	54.0
	1.0	44.2		10.0	17.4

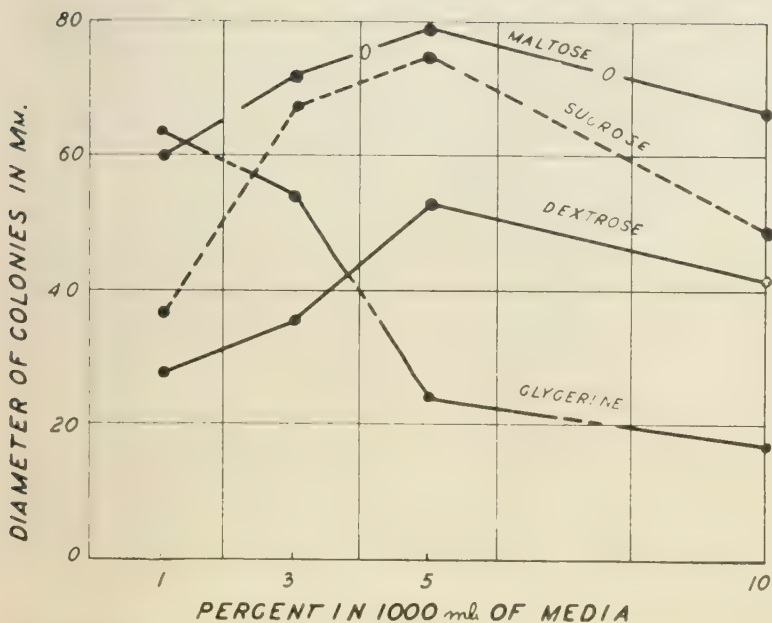


Figure 7--The influence of various sources of carbon upon the growth of *Verticillium V-10* after 15 days of growth in Czapek's agar.

colonies as the concentration of carbon increased. Poorest growth was observed on media containing ammonium sulphate as the source of nitrogen, with very little difference among the other four sources (Table 17 and Figure 8).

Since no information was available on the growth response to different temperatures, the fungus was planted on Czapek's agar medium and subjected for 15 day periods to temperatures of 5, 8, 10, 15, 20, 23, 25, 33, 35 and 40 degrees C. The growth responses at these temperatures were 3.0, 7.0, 23.0, 37.0, 55.1, 67.1, 64.4, 47.1, 18.4, 10.0 and 3mm., respectively (Figure 9). The optimum temperature seemed to be about 23°C.

Toxin Production by *Verticillium V-10*

Opinions differ concerning the cause of wilting in plants affected with *Verticillium*. Lawrence (1912), Whetzel and Rosenbaum (1912), Czarnecki (1923), and Van der Meer (1926) considered that thrombi in the vessels consisting of mycelium, gum-like substances, and tyloses cause wilt and desiccation of leaves. Bewley (1922), Rudolph (1931), and Dowson (1923) have argued against the

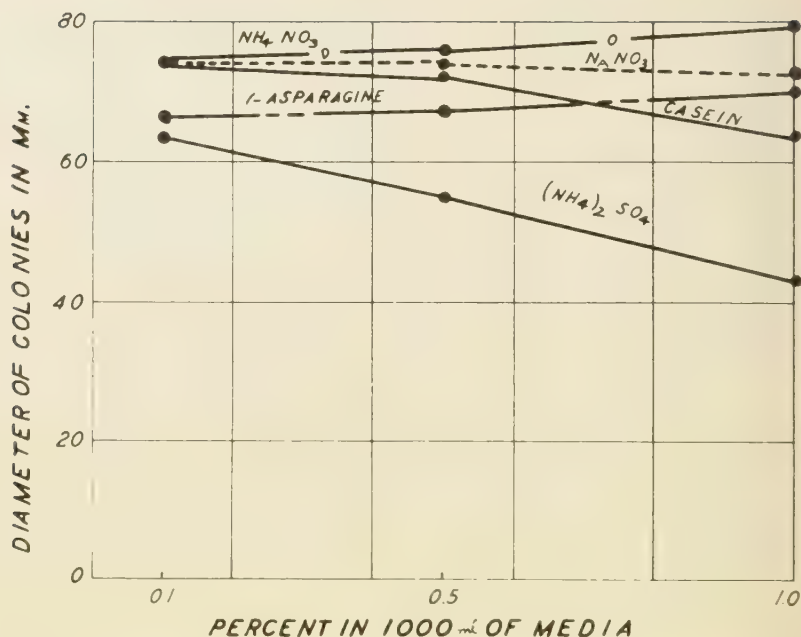


Figure 8—The influence of various sources of nitrogen upon the growth of *Verticillium* V-10 after 15 days incubation on Czapek's agar.

thrombosis theory, since it has been observed that many plants exhibiting severe wilt symptoms were found to have very few hyphae in the vessels, whereas some plants exhibiting only slight wilt symptoms showed an abundance of mycelium in the vascular elements. The author has noted several cases in which only slight discoloration of the sapwood was present, yet the foliage of a large branch was completely wilted.

Some evidence is at hand that *Verticillium* produces a metabolite which is largely responsible for wilt symptoms. Bewley (1922) found that *Verticillium* isolated from tomato plants, when cultured in Dox's sterile solution, produced a toxin which caused a wilt of tomato cuttings subjected to the filtrate. He also found that the toxin was water soluble, precipitable by ethanol, and thermostable. Dowson (1923) obtained results similar to those of Bewley when he used *Verticillium* isolated from Michaelmas daisies. Others who were of the opinion that *Verticillium* produces a toxin are Rudolph (1931) and Dufrenoy (1927). Rankin (1914) and Boyce (1938) have speculated that the maple wilt fungus produces a toxin which might be responsible for wilt. Records of actual experimentation using *Verticillium* isolated from maples, however, have not been found. Accordingly, one of the

projects of this work was to determine under what laboratory conditions this fungus could produce a toxin.

Following the studies by Howard (1941,) Feldman, *et al.*, (1950), Zentmyer, *et al.*, (1946) and Caroselli (1940) of various toxin-producing fungi, experiments were designed along similar lines. Various liquid media previously autoclaved at 114° C. for 30 minutes, and inoculated with a 3 mm. disc of *Verticillium* were subjected to still and agitated conditions and incubated for different periods of time. A filtrate from these cultures was obtained through a Buchner funnel with No. 50 Whatman filter-paper. Toxin production was determined by presence of wilt on young tomato cuttings (10 in each test) after a 24-hour period of insertion in the filtrate. If no wilt occurred within this period, the solution was presumed to be free of toxin. The toxic effects of the filtrate were also tested on tomato plants having the root system intact. The stems of cuttings were cut with a sharp scalpel under water prior to insertion in the small vials containing the filtrate. All tomato plants used in these tests had at least two well developed secondary leaves. The toxicity of the filtrate was further tested on maple cuttings (see Plate 6 & 7). Con-

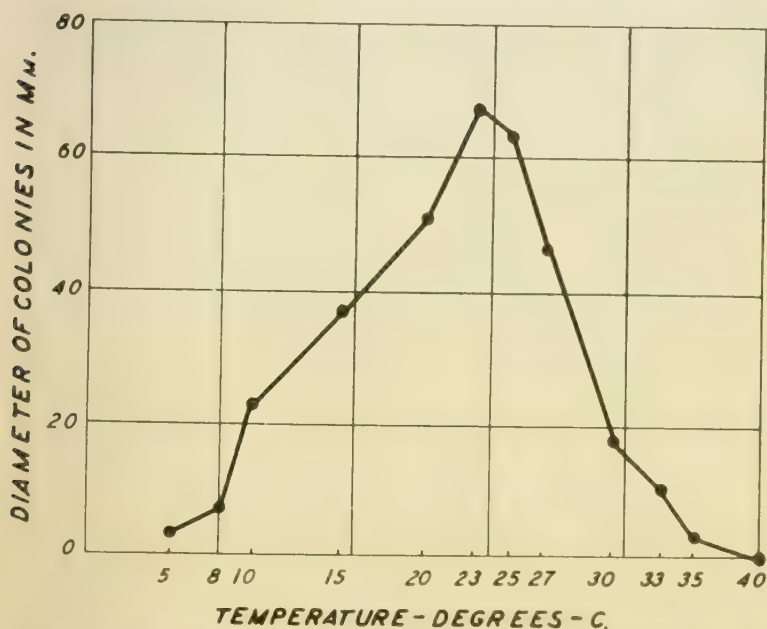


Figure 9—The influence of temperature upon the growth of *Verticillium V-10* after 15 days of incubation on Czapek's agar. It should be noted that the inoculum disc was 3 mm. in diameter.



Plate 6—Wilting of maple cuttings caused by the toxic filtrate produced by *Verticillium V-10* after growth for 9 days in agitated Richard's medium.

tol plants were inserted in medium previously autoclaved. In all cases, the pH of the test solution was adjusted to that of the sterile medium

The data in Table 18 show that at least a 30-day incubation period is needed for the production of a good titre of toxin—that is, one which causes both stem and leaf reaction. A high titre of toxin was produced by the fungus when grown in medium II for 30 days. In subsequent tests using this medium and a 30-day incubation period, it was found that in some cases injury to the test plants was confined primarily to the stem, whereas in other cases both stem and leaf injury occurred. Stem injury was in the form of constriction near the leaf petioles and flaccidity of the stem, whereas leaf injury was evident as curling and browning of the margins of the leaves and slight necrosis between the veins.

It had been noted by Feldman, *et al.*, (1950) that agitation of cultures reduced the time for production of toxin by *Geratostomella ulmi* Buisman. In order to find out if agitation would expedite toxin production by *Verticillium*, 250 ml. Erlenmeyer flasks containing 50 ml. of medium II inoculated with V-10 were placed on a shake table operating at forty-eight 4-inch strokes per minute. At the end of 1, 3, 5, 9, 12, and 15 days of shaking, the flasks were removed and tested for toxin. It was found that although toxin sometimes appeared at the

end of 3 days, the maximum titre was reached after 12 days of shaking. Beyond this period of agitation, there was no difference in the potency of the toxin produced.

Similar to the still cultures, shake cultures also produced a toxin which caused only stem injury in some instances and both stem and leaf damage in others. It has been observed that the toxin causing stem injury was produced in more instances than was the toxin which caused both stem and leaf wilt.

NATURE AND PROPERTIES OF THE TOXIN

Inasmuch as isolate V-10 was found to be capable of producing a toxin, the next phase in the experimentation was to determine, if possible, the nature and properties of the toxin.

The Relation of a Polysaccharide Fraction to Wilting

In previous tests it was observed that two types of wilt symptoms often occurred on young tomato plants after the cut stems were inserted into the toxic filtrate. Since both stem and leaf symptoms were not consistently caused by the



Plate 7—Tomato cuttings after 24 hours in the toxic filtrate produced by *Verticillium* V-10 after growth for 9 days in agitated Richard's medium.

Table 18 — Toxin-Production by *Verticillium V-10* in Various Liquid Media at Different Periods of Still Incubation.

Media*	Percent of Wilt**							
	5-Day Extraction Period		10-Day Extraction Period		20-Day Extraction Period		30-Day Extraction Period	
	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf
A	0	0	0	0	30	20	30	30
B	0	0	0	0	30	20	30	20
C	0	0	0	0	10	0	20	20
D	0	0	0	0	20	20	20	10
E	0	0	0	0	30	10	40	30
F	0	0	0	0	20	10	30	20
G	0	0	10	0	10	10	70	40
H	0	0	20	20	30	10	100	90
Richards***							50	50
Richard-C***							60	40
Leonian***							60	50
Leonian-C***							0	0
Czapek***							10	0
Czapek-C1***							50	50
Czapek-C2***							50	30
Czapek-C3***							70	60

*Formulae for all media are listed in Table 16.

**Each calculation represents 10 tomato cuttings.

***Only 30-day extraction period tested.

toxic filtrates, it was believed that the toxin might be composed of at least two fractions. Zentmyer *et al.* (1946) and Hodgson *et al.*, 1945, 1947, and 1947a isolated a polysaccharide from cultures of *Ceratostomella ulmi* and the crown-gall bacterium, respectively, which caused stem injury to tomato plants. In view of these findings, experiments were undertaken to determine whether or not *Verticillium* also produced a complex carbohydrate which could induce wilt of tomato plants. Experiments were designed to gain some information on such factors as yield of polysaccharide, wilt induced by the polysaccharide, and the relation of pH of the medium and growth of the fungus to polysaccharide production.

A carbohydrate was isolated from a 50-ml. aliquot of the Richard C. culture filtrate which was adjusted to the original pH of the medium. Fractionation was carried out at 30° C. *in vacuo*. When the volume of the medium had been reduced to about 10 ml., 28 ml. of 95 percent ethanol were very slowly added to the medium, which was kept constantly agitated. The carbohydrate usually was precipitated immediately and was removed by pouring off the supernatant liquid after the mixture had been kept in the refrigerator for 24 hours at 0 to 1° C. The carbohydrate was purified by redissolving in distilled water and reprecipitating with an excess of ethanol. This fraction was then added to enough distilled water or sterile medium to make up the original volume. The potency of the carbohydrate was judged by its ability to induce wilt of young tomato cuttings at the end of 24 hours. Since a maximum amount of carbohydrate could be produced within 7 to 9 days in shake cultures rather than 15 days in still cultures, the former method was employed. That this substance was a polysaccharide was ascertained by its positive reaction with Somogy's reagent. A positive test could

be attained only after 120 minutes hydrolysis with 0.1N HCl at between 70 and 80°C.

There was an apparent relationship between stem wilt and the amount of polysaccharide present in the medium (Figure 10). Severe stem injury was observed when the cut ends of plants were submerged in media having 2.0 to 2.5 mg. per cc. of polysaccharide.

The weight of the mycelial mat appeared to have no effect on the production of the polysaccharide. As shown in Table 22, the maximum production of the carbohydrate occurred after a 7-day shake period, at which time the mycelial mat weighed 193.0 mg. Longer periods of shaking did not stimulate the fungus to produce more polysaccharide, even though the weight of the mycelial mat increased.

It was noted during these tests that occasionally the affected stems of test plants would regain their turgidity after they were placed in distilled water. It seemed that this toxic fraction did not cause permanent injury. In order to determine whether affected plants could regain their turgidity, an experiment was conducted in which tomato cuttings were inserted in unused media containing the polysaccharide in concentrations of 0.5 to 3.0 mg. per ml. Five plants were used in each test. After a 24-hour exposure test period, all plants were taken out of the various media and placed in distilled water. All plants affected by the 0.5 mg. per ml. series recovered, whereas none of the other affected plants regained their turgidity.

Since it had been observed that the pH of the medium generally reached 6.0 to 6.4 at the end of a 7- to 9-day shake period, at which time the largest amount of polysaccharide was produced, it was believed that there might be a relation between pH and polysaccharide production. In order to determine if polysaccharide production was the result of a growth response of the fungus to pH of the medium, the fungus was grown in media having 4.0, 5.0, 6.0, and

Table 19 — Relation of Mycelial Growth to Polysaccharide Production in Shake Cultures.

Incubation Days	No. Flasks Used*	pH	Mycelial Weight (Mg.-cc.)	Polysaccharide (Mg.-cc.)
3	16	5.50	101.0	Trace
4	6	5.80	168.0	.81
5	12	6.34	185.0	1.01
6	4	6.40	173.0	1.17
7	10	6.70	193.0	2.10
9	4	7.20	221.1	2.00
11	2	7.30	252.0	2.00
15	12	7.21	270.0	1.94
17	4	7.10	291.0	1.90
19	2	7.23	284.0	1.63
21	2	7.30	296.0	1.54

*Contents of these flasks were pooled.

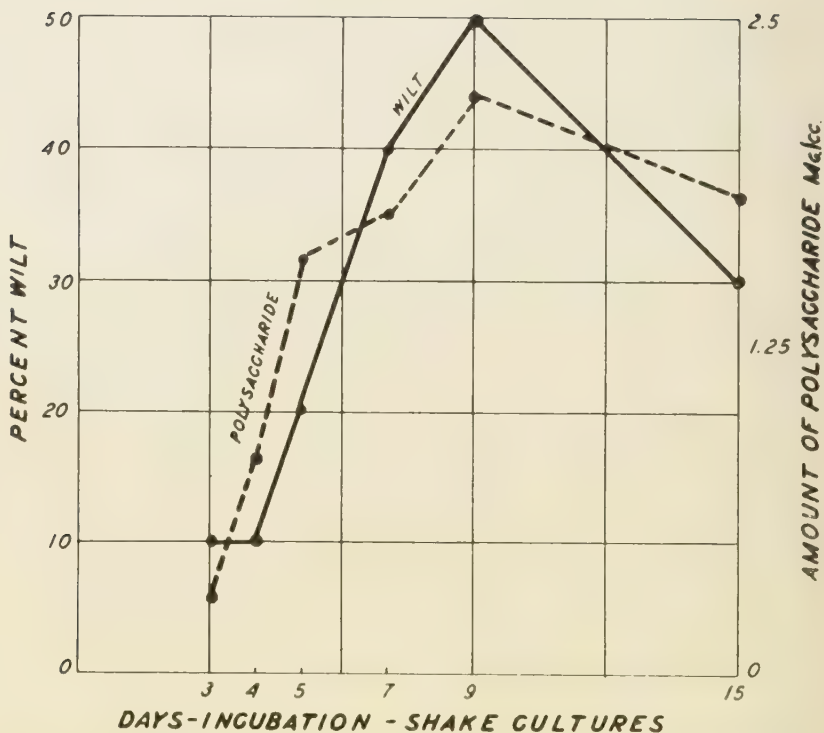


Figure 10—Relation of the degree of wilt in tomato cuttings to the amount of polysaccharide produced by *Verticillium V-10* in liquid medium H constantly agitated.

7.0 pH levels. The medium was buffered with a citric acid buffer-solution, as described by Gortner (1938), in order to help reduce pH fluctuations. In order to attain a pH of 4.0 and 5.0, citric acid HCl was added to the medium, whereas citric acid NaOH was added to reach the desired pH levels of 6.0 and 7.0. At the end of 15 days of shaking, the polysaccharide fraction of the toxin and the quantities of this complex carbohydrate in media having a pH of 4.0, 5.0, 6.0 and 7.0 were 2.2, 2.01, 2.1 and 2.1 mg. per ml., respectively. Only slight increases in pH occurred in the media buffered at pH of 4.0 and 5.0. These tests show that the production of polysaccharide by the fungus is not associated with changes of pH in the medium.

Properties of the Alcohol-Soluble Fraction

When the alcohol-soluble fraction was evaporated, a gum-like substance re-

mained. Tomato plants placed in a solution containing this gummy substance exhibited wilt of the leaves. Both stem- and leaf-wilt symptoms, however, were observed when this material was dissolved in a water solution containing the polysaccharide. In all cases, solutions were made up to the original volume of the filtrate from which the various fractions were isolated.

Extractions of the alcohol-soluble concentrate were made with ethyl ether. After being in contact with the concentrate for about 24 hours, the ether was poured into another glass receptacle and allowed to evaporate. Occasionally a very minute quantity of grayish substance was observed, which was soluble in water. Tomato cuttings placed in solutions containing this substance usually exhibited no outward symptoms of injury. On one occasion, however, slight stem flaccidity was observed in a group of five test plants.

The ether-insoluble, alcohol-soluble concentrate, when placed in distilled water, produced leaf wilt of tomato cuttings which were inserted therein. It appears that the chemical which causes leaf wilt of tomato cuttings is soluble in alcohol and insoluble in ether.

Ether Extractions of the Culture Filtrate

Tests were also conducted in which ether extractions were made of the toxic filtrate which had not been subjected to alcohol extraction. Shortly after ether was added to test tubes containing the filtrate, two distinct layers were formed. The top layer was very cloudy and the bottom layer was clear. The two layers were separated and the ether was evaporated at room temperature. The residue of the ether-soluble fraction was redissolved in unused medium and tested for its phytotoxicity. Stem wilt was noticed in tomato plants placed in this solution, which apparently contained a polysaccharide. When the cut ends of tomato plants were placed in a solution containing the ether-soluble concentrate to which was added the alcohol-soluble fraction, both stem and leaf wilt ensued.

Relation of Thiourea to Wilt

These investigations thus far have shown that wilt symptoms on test plants were caused by a polysaccharide and an alcohol-soluble fraction, the former causing stem injury and the latter leaf wilt.

Ovcarov (1937) found that thiourea could be produced by *Ferticallium albo-atrum* Reinke and Berth. when grown in different liquid media containing either casein, peptone or ammonium nitrate. He also found that weak solutions of thiourea were capable of producing wilt in the leaves of tomato plants within 48 hours. In view of this work, investigations were undertaken in an effort to determine if there was any relation between the alcohol-soluble fraction and thiourea. Studies were made of both still and shake cultures grown in medium H

containing either casein, ammonium nitrate, ammonium sulphate, l-asparagine, gelatine, sodium nitrate, edestin, or peptone as the sources of nitrogen. The cultures were incubated for different periods at room temperature. The presence of thiourea was tested by Klein's method suggested by Ovcarov (1937), as well as by Grote's (1931) method as modified by Nicholes and Herrin (1941). Best results were obtained by adding 15 drops of Grote's reagent to about 25 ml. of the toxic filtrate; a positive reaction was obtained within 24 hours. Checks using sterile nutrient media were compared in every experiment.

The results as tabulated in Table 20 show that a positive test for thiourea was obtained in certain cases when the fungus was grown in the presence of all sources

Table 20 — The Effect of Nitrogen Sources and Time of Incubation (still and shake) upon the Production of Thiourea.

Nitrogen Source	No. of Days		No. of Flasks		Average % Wilt of Stem and Leaf
	Still Cultures	Shake Cultures	Tested	Positive for Thiourea	
NH ₄ NO ₃		3	4	0	0
		4	4	0	0
		5	2	0	10
		6	5	0	10
		7	4	0	20
		8	4	0	30
		10	2	0	30
		12	4	3	80
		13	14	6	80
		15	2	1	70
		20	2	0	40
			2	0	90
			2	2	40
(NH ₄) ₂ SO ₄	30		2	0	50
	60		2	0	70
	90		2	3	10
Asparagine		12	8	0	10
		5	2	0	10
		6	2	0	10
		7	2	0	20
		9	2	0	20
		12	12	5	70
		15	2	1	80
		20	2	0	40
		25	2	1	90
		30	3	1	90
	20		2	2	80
	30		2	0	40
Gelatine		3	4	0	0
		5	2	0	0
		7	2	0	10
		12	4	2	80
		15	4	1	80
Peptone		15	4	2	80
Edestine		15	4	1	70
Casein		15	6	2	80
	30		10	4	80
NaNO ₃		3	4	0	0
		5	2	0	0
		7	4	0	0
		9	2	0	0
		12	2	0	0
	30		4	0	0

of nitrogen except sodium nitrate. Regardless of the length of period of shaking or standing in the incubation process, cultures grown in the presence of sodium nitrate never gave a positive test for thiourea. With the other nitrogen sources, thiourea appeared at the end of 12 to 15 days when the culture was shaken, and after 25 to 30 days of still incubation. Filtrates of all test flasks did not give a positive test for thiourea. Assuming that a minimum of 12 shake days and 20 still days were needed for thiourea production, a positive test for this chemical was noted in 38.8 percent and 40 percent for these incubation periods, respectively. These figures are based upon the occurrence of thiourea in the presence of all sources of nitrogen, except sodium nitrate. It was interesting that from a batch of flasks having identical medium, inoculum and incubation period, thiourea could be expected to be produced in about 40 percent of the flasks.

The factors responsible for inconsistent production of thiourea have not been determined. Since it has been observed that *Verticillium* is capable of sectoring in culture, there is always a possibility that a colony of uniform appearance may contain saltant hyphae which may grow well in liquid media but are unable to produce thiourea. We have observed that a white colony isolated from a white sector of a V-10 colony never produced thiourea, although it consistently produced a polysaccharide.



Plate 8—Sugar maple seedlings prior to injection with a concentrate dose of toxin produced by *Verticillium* V-10.

Plate 9—Sugar maple seedlings displaying foliar injury two weeks after injection with toxin produced by *Verticillium* V-10.

Filtrates giving a positive test for thiourea were all found to contain the polysaccharide fraction in varying amounts. On the other hand, a polysaccharide which was isolated consistently from cultures regardless of the nitrogen source was not always accompanied by production of thiourea. This situation explains why a difference in the type and severity of wilt may occur on tomato seedlings used in toxin-assay tests.

Wilting of the leaves caused by the thiourea fraction was never reversible when plants were placed in distilled water. Similar injury was noted on tomato plants placed in solutions having known quantities of chemically pure thiourea. In these tests, marginal leaf injury was observed in plants inserted in solutions containing as low as .09 percent thiourea.

In an attempt to isolate the thiourea fraction from diseased tomato plants, plant parts were dried, crushed, placed in stoppered flasks containing 70 percent alcohol, and kept at 100° C. for three hours. After this extraction period, the alcohol was evaporated at room temperature and the residue was placed in the distilled water. This mixture was cleared in animal charcoal and tested for thiourea. Thus far, only 1 test out of 10 gave a very slight positive test for thiourea. More tests are needed before any conclusions can be drawn.

Miscellaneous Tests

Failure of the toxic filtrate to give a positive reaction with Millon, Biuret, and xanthoproteic reagents indicated that no protein was present. The urease test suggested by Cole (1920) demonstrated that no urea was present (see Table 21).

That the toxic components of the filtrate are thermostable has been demonstrated by their ability to induce wilt even after being heated at 100° C. for 10 minutes.

The toxic filtrate, when injected into young trees, was capable of causing wilt symptoms which were similar to those occurring on trees inoculated with *Verticillium* (Plates 6, 8 and 9).

Table 21 — Results of Test for Urea in Cultures of *Verticillium V-10* Grown in Media with Different Nitrogen Sources.

Nitrogen Source	pH of Pooled Toxin*	No. of Flasks	Test for Urea
NaNO ₃	6.9	7	—
Asparagine	7.1	12	—
Gelatine	7.2	6	—
Control (NaNO ₃)	5.2	2	—
Control (Asp.)	5.2	2	—
Control (Gel.)	5.2	2	—
Control (Urea)	5.2	2	—

*pH after growth of fungus, adjusted to 5.2 prior to testing.

These experiments show that at least two toxic components are produced by the maple wilt fungus, *Verticillium* sp., when grown in liquid media under laboratory conditions. One is a carbohydrate and the other a thiourea compound. The carbohydrate fraction *per se* caused stem wilt in about 50 percent of the plants tested and slight leaf wilt in 20 percent. Thiourea, on the other hand, was found to cause leaf wilt in about 60 per cent of the test plants, and stem injury in 20 percent. A combination of the two fractions, however, caused as high as 90 percent stem and leaf wilt. Leaves wilted in the presence of the polysaccharide fraction did not display typical marginal necrosis or other symptoms that are associated with the thiourea fraction. Wilt in this case may have been caused by an interference with the water economy of the cuttings and probably resulted from partial obstruction of the conducting elements by the polysaccharide. Hodgson *et al.* (1947) demonstrated that polysaccharides and other polymers may interfere with the translocation of water in plants.

If the toxic entities as produced under laboratory conditions are similarly produced in a diseased tree, then we can assume that toxins play an important role in pathogenesis and thus account for the multiplicity of symptoms expressed by trees infected with *Verticillium* sp.

The Amount of Liquid Uptake by Test Plants As Influenced by the Toxin Produced by *Verticillium* Sp.

While demonstrating the phytotoxic effects of the filtrate, it was observed that, after wilt occurred, the amount of residual liquid in tubes containing the toxin appeared to be slightly greater than the amounts present in tubes containing either the check medium or water. It was conjectured that the toxin affected the total uptake of liquid by these plants. Therefore, an experiment was conducted to compare differences of total liquid uptake by tomato cuttings placed in the toxic filtrate and others in a control medium. The literature is lacking in information on the water economy of *Verticillium*-wilted maple trees. Bewley (1928) subjected diseased cucumber plants in different states of wilt to a temperature of 25°C. in a greenhouse. Some of the plants were shaded, others were not. He reported that a greater number of plants had recovered in the shaded house. Bewley expressed the opinion that, although temperature is an important factor, shade is valuable since it reduces transpiration, which in turn prevents upward translocation of large amounts of toxic products excreted by the pathogene.

In all experiments, the filtrate used was produced by the V-10 isolate grown in medium H at room temperature during a 15-day period of shaking. In order to determine whether gases were present in xylem elements, affected plants were cleared in an alcohol-clove-oil mixture before being examined microscopically.

Uniform, 3-week-old, greenhouse-grown Stone tomato plants with two well

developed secondary leaves were used to ascertain the effect of the toxin complex on total liquid uptake. The toxic filtrate and check medium were put into separate series of U-tubes. One end of the tube contained a tight fitting, tapering, one-hole cork. Stems of tomato seedlings were cut under water with a razor blade. The cut end was inserted into the corked stoppered end of the tube. An air-tight seal was insured by placing a substantial quantity of lanolin around stem and cork. In order to prevent dust from falling into the filtrate and also to help reduce loss of liquid by evaporation, the other end of the U-tube was sealed with a small, absorbent-cotton plug. This leg of the tube was marked to indicate the initial height of the filtrate column. Total liquid uptake was determined by measuring the water loss in the tube. Hourly readings were recorded until a negligible amount of water loss was noted after complete wilting of the test plants occurred. Before starting, the pH of the toxic filtrate was adjusted to that of the check. Tests were also conducted using a dilution series of 75 percent, 50 percent and 25 percent toxin filtrate. Each test was repeated at least twice and conducted under room temperature conditions. Twenty-five plants were used for each of the three tests of the medium with 100 percent toxin; the same number of plants was used for the first test of each dilution of 75 percent, 50 percent and 25 percent toxin, whereas only 10 plants were used for the second and third trials.

Over a 13-hour period the toxin produced by *Verticillium* sp. reduced the total liquid uptake by test over check plants (Table 22 and Figure 11). Inhibition of liquid uptake decreased, however, when smaller quantities of toxin were present. Three tests conducted for each dilution showed little difference in total loss of liquid. The difference between the first and second runs may possibly be attributed to temperature, since it was higher when the second run was conducted.

The time when the first perceptible symptoms of wilt occurred varied with each group of tomato seedlings subjected to the various concentrations of toxin. Curling of the leaf margins was noted at the end of 6, 8, 9 and 10 hours for the 100, 75, 50, and 25 percent toxin, respectively. Complete wilting of foliage occurred at the end of 12 hours on those plants placed in the 100 percent and 75 percent toxin and 13 and 16 hours for seedlings in 50 percent and 25 percent toxin, respectively. There was no evidence of stem necrosis even under conditions of severe wilt of the foliage. No wilt was noted on any of the control plants.

Toxin produced by the maple wilt *Verticillium* appears to inhibit total liquid uptake in tomato plants. The role of this toxic metabolite relative to its effect on liquid movement in these plants is still obscure.

Upon completion of the various tests, bacteria (cocci) were observed in both the check medium and the toxic filtrate. Microscopic examinations failed to disclose any significant difference in the number of bacteria growing in the various

Table 22 — Effect of Toxin on Total Liquid Uptake.

		Uptake per Hour (ml.)										
		2	4	5	6	7	8	9	10	11	12	13
Check Medium	1st Run	.146	.152	.131	.091	.089	.076	.063	.064	.055	.052	.051
	2nd Run	.150	.152	.126	.108	.089	.087	.078	.163	.058	.057	.058
	3rd Run	.148	.152	.130	.107	.089	.090	.070	.063	.057	.057	.055
Toxin (100%)	Average	.148	.152	.129	.099	.089	.081	.070	.063	.057	.055	.055
	1st Run	.122	.119	.068	.040	.051	.016	.012	.010	.015	.013	.012
	2nd Run	.125	.119	.094	.064	.026	.035	.023	.013	.011	.007	.012
Toxin (75%)	3rd Run	.123	.119	.081	.052	.039	.024	.019	.016	.013	.016	.012
	Average	.123	.119	.081	.052	.038	.025	.018	.013	.013	.012	.012
	1st Run	.123	.122	.101	.089	.071	.054	.041	.028	.018	.012	.009
Toxin (50%)	2nd Run	.123	.121	.094	.076	.056	.045	.031	.023	.012	.022	.021
	3rd Run	.122	.121	.097	.081	.063	.037	.034	.024	.023	.019	.019
	Average	.123	.121	.097	.082	.063	.045	.035	.025	.017	.017	.016
Toxin (25%)	1st Run	.128	.124	.102	.092	.088	.073	.064	.043	.033	.027	.017
	2nd Run	.133	.131	.098	.093	.081	.067	.046	.039	.035	.027	.017
	3rd Run	.131	.129	.101	.094	.086	.071	.045	.048	.034	.026	.022
Toxin (23%)	Average	.130	.128	.100	.093	.085	.070	.045	.043	.034	.027	.018
	1st Run	.136	.135	.104	.089	.099	.071	.052	.051	.037	.032	.032
	2nd Run	.135	.134	.106	.087	.093	.073	.054	.051	.035	.033	.031
Toxin (23%)	3rd Run	.136	.135	.105	.089	.091	.073	.053	.050	.041	.033	.034
	Average	.136	.135	.105	.089	.096	.073	.053	.051	.038	.033	.033

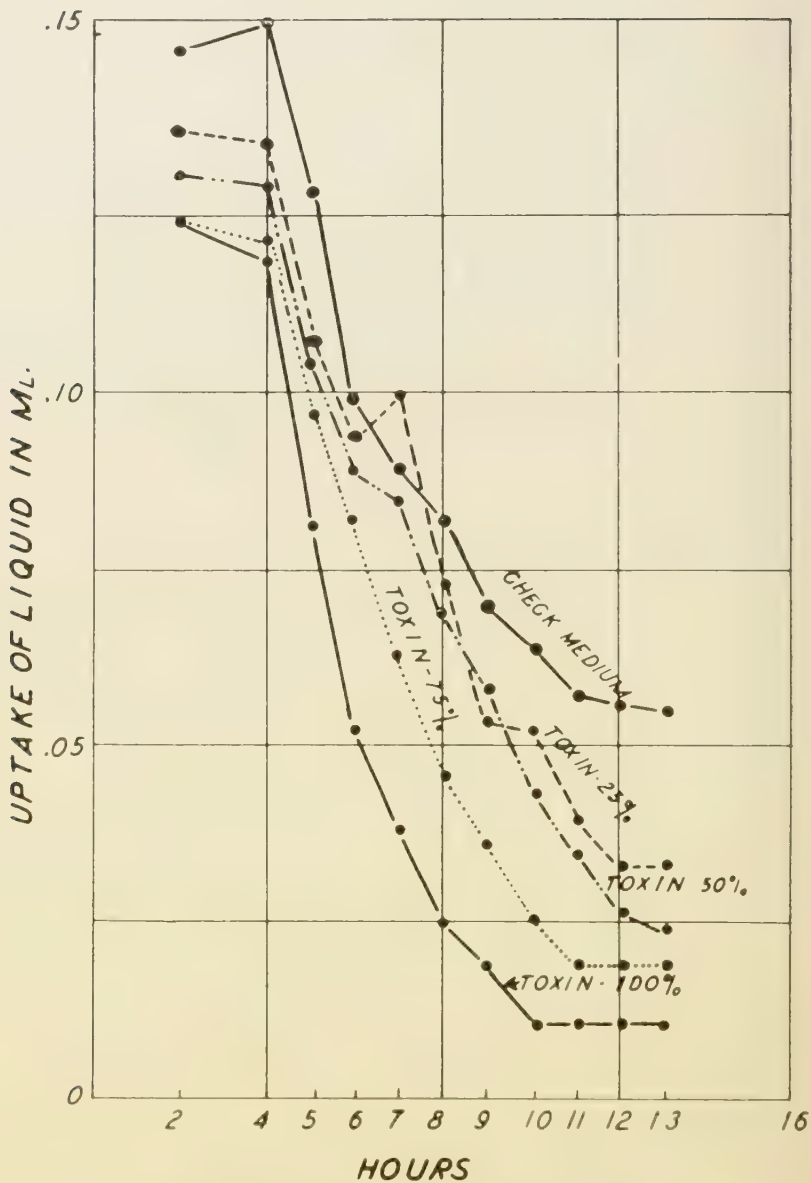


Figure 11—The effect of different concentrations of toxin upon total liquid uptake by young tomato cuttings. Average of 3 runs.

solutions used in the experiments. These bacteria seem to have no influence on the inhibition of liquid movement by plugging the vascular tissues.

The reduction of loss of liquid by the presence of gases in the plant tissues has also been the subject of speculation. Examinations of affected tomato plants did not reveal gas embolism.

Free-hand sections made of the affected tomato cuttings were examined and found free of any gum-like substances. Also, no appreciable difference in the amount of tyloses present in the vessels of check and wilted plants could be detected.

The difference in loss of water in these plants cannot be attributed to the pH of the check medium or the toxic filtrate, since the pH of the latter was adjusted to the pH of the former. What, then, is the mechanism responsible for the differences in liquid uptake?

An impeding of the water movement in these plants by the polysaccharide could not be completely responsible for wilt, since the leaves exhibited symptoms similar to those produced by the fraction containing thiourea. It appears that the toxic filtrate is composed of at least two fractions — one, thiourea, which is translocated to the leaves where it may upset the differential permeability of the parenchyma cells so that they do not function as osmotic units, and second, a polysaccharide, probably interfering with the normal translocation activities in the xylem elements.

EFFECT OF CHEMICALS ON THE FUNGUS AND THE TOXINS

Numerous chemicals were tested for their fungistatic and fungicidal properties against *Feticillium* sp. These tests were conducted by placing a 3 mm. disc of nutrient agar with a mat of mycelium growing thereon in 1:1000 and 1:10,000 solution of the candidate chemicals for about 24 hours. The mats were rinsed in three changes of sterile distilled water and then placed in petri dishes containing Czapek's agar medium. The effectiveness of the chemicals was based on the ability of the fungus to grow out from the treated disc onto the adjacent nutrient medium. Of the 150 chemicals tested, those which displayed some inhibitory or fungicidal properties are listed below:

copper sulphate
acriflavine (neutral) N.F. IX
acriflavine hydrochloride
acriviolet
brilliant green B crystals
gentian violet U.S.P. XIII

proflavine N.F. IX
quinine bisulphate
quinine sulphate
hydroquinone
8-hydroxyquinolin
8-hydroxyquinolin sulphate

helione orange
malachite green

8-hydroxyquinolin benzoate
salicylic acid
urea

Of the 17 chemicals listed above, whose phytotoxicity to succulent maple cuttings was determined as shown in Table 23, only 6 caused no injury.

Table 23 — Phytotoxicity of Certain Chemicals at 1:1000 to Maple Cuttings in Full Leaf under Laboratory Conditions.

No Injury	Slight Injury	Severe Injury
Acridlavine	Helione Orange	8-Hydroxyquinoline
Hydrochloride	Urea	Sulphate
Proflavine N.F. IX	Acridlavine	8-Hydroxyquinoline
Sulfate	(Neutral) N.F. IX	Benzoate
Quinine Bi-sulphate		Salicylic Acid
Quinine Sulphate		Acridviolet
Hydroquinone		Brilliant Green
8-Hydroxy-quinoline		Malachite Green
		Gentian Violet
		U.S.P. XIII
		Copper Sulphate

Howard, (1941) and Caroselli, (1940) found that a mycotoxin produced by *Phytophthora cactorum* could be antidoted by an organic dyestuff known as helione orange. Successful antidoting of the toxin produced by the Dutch elm disease fungus was accomplished by Zentmyer, *et al.* (1946), and Feldman, *et al.* (1950). A similar approach to the maple wilt disease was also undertaken and it has been found that hydroquinone at a dilution of 1:9000 will neutralize the toxin formed *in vitro* by *Verticillium* (Plate 11). Other chemicals which exhibited some antidoting properties were acridlavine hydrochloride and proflavine N.F. IX.

A COMPARATIVE STUDY OF 10 PATHOGENIC STRAINS OF *VERTICILLIUM* SP. ISOLATED FROM WILTED MAPLE TREES

Cultural differences and morphological changes in cultures of *Verticillium albo-atrum* Reinke and Berth. when grown on nutrient media have been reported by Carpenter (1918), Zimm (1918), Chaudhuri (1925), Rudolph (1931), Baines (1945), Schneider (1948), and Presley (1941, 1950). The ability of this species to produce microsclerotia under laboratory conditions has resulted in numerous controversies as to whether there should really be two different species, *V. albo-atrum* and *V. dahliae* Kleb. The latter was considered to be distinct from the former and was given specific rank by Klebahn (1913) because of its ability to produce microsclerotia and the complete absence of such structures in *V. albo-atrum*.

It was not the purpose of the following experiments and studies to determine what species of *Verticillium* is responsible for the wilt of maples, but to point out that the maple wilt fungus produces different segregates or strains which develop

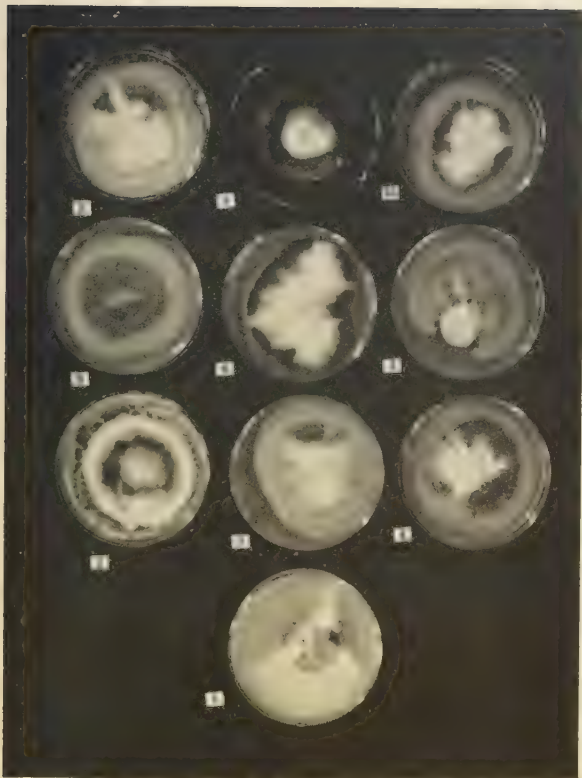


Plate 10—Variation in mycelial growth and sporulation of *Verticillium* isolates V-1 to V-10 growing on Czapek's nutrient medium.

in the laboratory as a result of common transfer procedures as well as from monoklonal isolations and to report certain studies upon these segregates for what bearing they may have upon the pathology and mycology of the fungus.

Description of Strains

Over the past few years, from 150 to 200 isolates were made from wilted maple trees—sugar, Norway, sycamore, red and Japanese maples—in New Hampshire, Vermont, Massachusetts, Rhode Island, Connecticut, New York, New Jersey, Pennsylvania and Delaware. Cultures were obtained by planting chips of discolored sapwood on malt agar or Czapek's agar. When these were all regrown on Czapek's agar medium and incubated at room temperature (about 20° C.), they

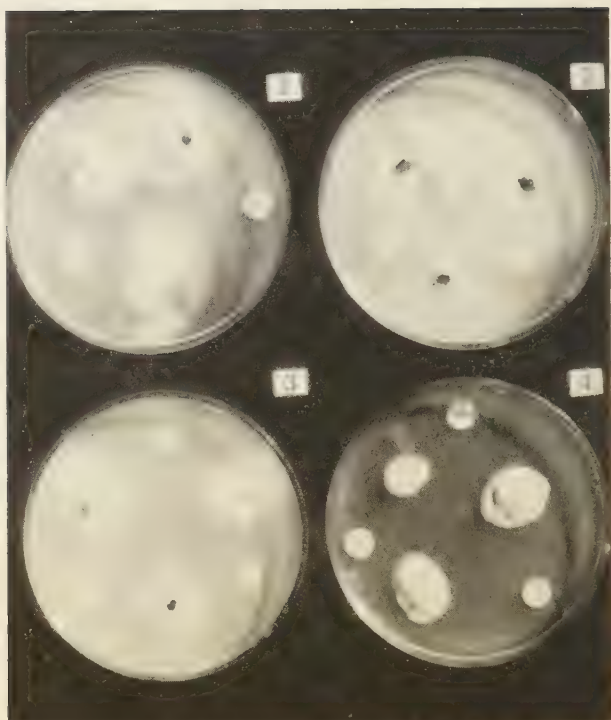


Plate 11—Inhibition of *Verticillium* V-1 and V-10 by a toxic metabolite produced by *Bacillus subtilis*. Note the inhibition of V-10 around the discs saturated with the metabolite in dishes 1 and 3, and V-1 in dish 4. Dish 2 is a control with no inhibition.

fell into 10 readily distinguishable groups or strains, designated for convenience from V-1 to V-10. Descriptions of these follow and are illustrated in Plate 10.

V-1 —The mycelium of these isolates tended to grow appressed to the surface of the medium and only rarely extended itself vertically. Microsclerotia, produced only rarely, were brown in color. No chlamydospores or black mycelium were noted. The medium beneath the colony was grayish in color. Compound conidiophores were present in abundance.

V-2 —Isolates of this group produced two types of mycelium: one submersed in the medium and black in color, the other a fluffy, light gray, aerial mycelium. Black microsclerotia and chlamydospores were produced in appreciable numbers. The Czapek agar medium beneath the colony exhibited a greenish shade. Conidia

were often produced on simple branchlets from the mycelium as well as on simple conidiophores.

V-3—This group formed a grayish, fluffy, intermediate mycelium; *i.e.*, a type between repent and aerial, slightly raised but nevertheless quite compact. No black mycelium was produced. Microsclerotia were black and produced in such large numbers that the medium beneath the colony was jet black in color. Chlamydospores were rarely observed and conidia were borne mostly on simple conidiophores.

V-4—These cultures produced for the most part a white mycelium closely appressed to the surface but with occasional light gray clumps. There was no sign of black mycelium. The colonies produced an abundance of black microsclerotia which gave the medium beneath a black color. Chlamydospores were rarely produced and conidia were borne on simple conidiophores.

V-5—The V-5 isolates were characterized by a dark gray, appressed, surface mycelium, generally with a small area of lighter gray in the center of the colony. Papillate elevations were scattered over the surface. The microsclerotia were black and abundant, imparting to the medium beneath the colonies a black color. Neither black mycelium nor chlamydospores were observed. Conidiophores were simple for the most part, only rarely compound.

V-6—This group of isolates developed a gray, cottony, aerial mycelium, and a black superficial or submersed mycelium the hyphae of which often interlaced to form knots. The production of microsclerotia and chlamydospores was rare. The medium below the colonies had a greenish sheen. Conidia were borne on simple conidiophores.

V-7—This category exhibited an abundance of fluffy, white, aerial mycelium, with no black at all, which here and there interwove into clumps like balls of yarn. Microsclerotia, when produced, were brown in color, giving the medium beneath a mottled, grayish appearance. Chlamydospores were noted only rarely. Conidia were borne on both simple and compound conidiophores.

V-8—The aerial mycelium was white; no black mycelium or chlamydospores were observed. Black microsclerotia were produced in abundance. The medium beneath the colonies was jet black. Conidia were borne mostly on simple conidiophores.

V-9—This strain had, except for a central white, appressed portion, a fluffy, fumose, aerial mycelium and a black, resting mycelium which gave a greenish color to the lower surface of the colonies. Chlamydospores were seen in all colonies examined, but microsclerotia were rarely produced. Conidia were borne primarily on simple conidiophores, but they were also found on branchlets of hyphal segments.

V-10—The mycelium of this group of isolates was white and somewhat appressed. No black mycelium was produced and chlamydospores only rarely. Black microsclerotia were formed in concentric rings. The medium beneath the colony was a pale black. Conidia were borne on simple conidiophores.

The foregoing strains were readily distinguishable and the recognizable characteristics were in general maintained with reasonable uniformity in continuing subcultures made by mass mycelial transfers. Rarely, it is to be noted, was there obtained from any culture of these 10 strains a variant subculture with a thick mat of entirely white mycelium and no microsclerotia or chlamydospores. After the second or third transfer from these, however, and with a sufficiently long incubation period, the colonies always reverted to the original type. This "matted-white type" was also produced rarely in cultures which were derived from a single conidium (see next to last column in Table 24).

Another variant may also be noted here—the "non-matted white variant" of the last column of Table 24; produced only from single conidia of V-6 and V-10 and never by mass mycelial transfers. The mycelial growth was thin, sparse, loose, more or less patchy, with only scant aerial growth, without clumps or knots or any interlacing hyphae, and with no obvious microsclerotia or chlamydospores. This variant retained its colony characteristics through all subsequent culturing and never reverted to the original condition.

If the two white variants are disregarded for the moment, it is interesting, to note that the 10 original strains may be segregated into three groups, as below —

A	B	C
(V-1 & V-7)	(V-2, V-6 & V-9)	(V-3, V-4, V-5, V-8 & V-10)
No black mycelium	Black mycelium present	No black mycelium
Microsclerotia	Microsclerotia	Black microsclerotia
brown but few	black, few to rare	present, abundant
Medium beneath	Medium beneath	Medium beneath
colonies grayish	colonies greenish	colonies black
Chlamydospores	Chlamydospores	Chlamydospores none
none or rare	present	or rare
Conidiophores in	Conidiophores simple	Conidiophores simple
part compound		

Monoconidial Isolates

In view of the apparent differences between the 10 strains and the reasonable constancy of cultural response by mycelial transfers, it was considered desirable to find out what would be the situation with monoconidial isolates—what sort of segregations would appear, and whether or not the same strains would appear or possibly some new ones. Spore suspensions prepared for each of the strains which

Table 24 — Cultures Produced by Monoconidial Isolates from Each of Ten Isolates of *Verticillium* sp. Procured from Infected Maple Trees.

Isolate	No. of Cultures Obtained	Number and type of colonies produced									Matted Non-White Mat-variant	
		V-1	V-2	V-3	V-4	V-5	V-6	V-7	V-8	V-9	V-10	Variant
V-1	27	21						6				
V-2	25		11				7			6		1
V-3	25			13		4					6	2
V-4	20				9				5		5	1
V-5	22			1		13					7	1
V-6	21		3				12			5		1
V-7	20	7						12				1
V-8	22				1				13		7	1
V-9	23		4				5			13		1
V-10	35					1			8		24	1

had grown for 21 days on Czapek's solid medium were diluted and dispensed evenly into petri dishes containing a thin layer of the same medium. After these spores had germinated, they were fished out and individually transferred to petri dishes containing approximately 15 ml. of Czapek's medium. These dishes were incubated for 20 days at 23° C. The resultant cultures are tabulated in Table 24.

There are several things of interest in this table. One is that the various conidia of every strain gave at least one other strain in addition to its own. Another is that conidia of every strain gave no more than 1 or 2 cultures of other strains, leaving out for the moment the 2 white variants. Only V-1 and V-7 (Group A) gave one other strain, each the other one. All the other strains produced two other strains each.

Another matter of interest is the proportions of the numbers of strains produced by the single conidia. In only two cases (V-2 & V-4) was the original strain reproduced in less than 50 percent of the total. With V-3, the proportion of the original to the others was slightly better than 1:1, and with the other strains the ratios varied from 3:2 to 3:1.

A fourth point is that a scrutiny of Table 27 will show that the products of the segregated conidia group themselves into A, B and C. Observe that the four figures for V-1 and V-7 (Group A) make a square; the eight figures for V-2, V-6 and V-9 form a rectangle horizontally and for V-6 and V-9 a vertical rectangle

Table 24A — Rearrangement of Table 24 with Strains by Groups.

	Group A		Group B			Group C					
	V-1	V-7	V-2	V-6	V-9	V-3	V-4	V-5	V-8	V-10	
V-1	21	6									
V-7	7	12									
V-2			11	7	6						
V-6			3	12	5						
V-9			4	5	13						
V-3						13	—	4	—	6	
V-4							9	—	5	5	
V-5						1	—	13	—	7	
V-8							1	—	13	7	
V-10								1	8	24	

interlocking with the preceding one (Group B); and that the 7, 7, and 6 figures, respectively, for V-3, V-5 and V-10, for V-4, V-8 and V-10, and for V-5, V-8 and V-10, form interlocking rectangles (Group C). Or, to represent the situation more graphically, examine the rearrangement in Table 24A of Table 24 along the foregoing group lines.

A fifth feature is the regularity of the results of microconidial growth (except for the two white variants) – the absence of important gaps in Table 24 and 24A and the lack of striking incongruities. Of course, there were only 150 to 200 isolates of the maple wilt fungus in the first place and from the 10 strains segregated from these, only 240 single conidia were isolated for the experiments. What the situation would be if more hundreds or thousands of conidia were germinated and grown from more hundreds of isolates from maples is conjectural. Further, what may be the meaning of the facts and groupings presented here is beyond the scope of this work.

Physiology of the Strains

In order to determine if there were other than morphological differences between the 10 strains, they were grown at different temperatures and upon media providing different sources of nitrogen and carbon. They were also tested for their ability to produce toxin and to induce disease in tomato plants and eggplants.

In all pertinent experiments petri dishes containing approximately the same amount of Czapek's solid medium were inoculated each with a 3 mm. disc of the fungus growing on the same nutrient medium. Care was taken to maintain the same thickness of the substrate in all cases since Chaudhuri (1923) reported that growth of a fungus may vary with the thickness of the medium. Cultures were incubated for 15 days and results were recorded as averages from 3 to 5 replicates.

RESPONSE OF THE 10 STRAINS TO TEMPERATURE

There is a difference of opinion in the literature on the affect of temperature upon the growth of *Fusicillium* sp. under laboratory conditions. Chaudhuri (1923) reported that the most luxuriant growth of *F. albo-atrum* on nutrient-agar media occurred at 22.5°C. Edson and Shapovalov (1920) found that this species grew best at 25°C. Bewley (1922) reported that the optimum temperature for growth of this same species was 23.3°C. It is possible that these workers may have been employing different biotypes of the fungus.

The 10 strains of our fungus were subjected to 11 temperatures from 5° to 35°C. The results showing the growth responses at the different temperatures are given by strains and by groups of strains in Table 25.

Table 25 — Influence of Temperature upon the Growth of 10 Strains of *Verticillium* sp. on Czapek's Medium over a 15-Day Period.

Temperature °C.	Size of Colonies (in mm.) of Isolates										Average of All Isolates in the Three Groups		
	V-1	V-2	V-3	V-4	V-5	V-6	V-7	V-8	V-9	V-10	Group A (V-1 & 7)	Group B (V-2, 6 & 9)	Group C (V-3, 4, 5, 8 & 10)
5	3.0*	3.0*	3.0*	3.0*	3.0*	3.0*	3.0*	3.0*	3.0*	3.0*	3.0*	3.0*	3.0*
8	4.0	5.0	6.0	6.5	5.5	5.0	6.5	5.0	5.0	5.0	5.2	5.0	5.1
10	16.0	18.5	22.0	23.0	21.5	16.0	22.0	21.0	19.0	23.0	19.0	17.7	22.1
15	30.0	33.0	34.0	30.1	33.4	30.1	35.9	34.0	33.2	33.0	32.9	32.1	32.9
20	41.0	41.1	50.2	50.3	43.3	46.6	50.3	44.1	45.5	55.1	46.1	45.5	48.6
23	56.1	61.0	60.4	61.0	65.7	59.3	66.0	65.1	61.3	67.1	61.0	60.5	63.8
25	56.5	60.1	60.1	61.5	56.3	60.0	54.0	55.2	60.3	60.4	55.2	60.1	58.7
27	43.9	47.8	46.0	46.3	46.0	44.0	46.4	45.2	42.1	47.1	45.1	44.5	46.2
30	12.1	23.9	17.9	18.1	17.0	12.3	17.4	16.7	24.3	18.4	14.7	20.1	29.3
33	4.0	8.0	8.1	8.0	7.6	4.0	8.0	7.9	8.0	10.0	5.8	8.8	8.3
35	3.0*	3.0*	3.0*	3.0*	3.0*	3.0*	3.0*	3.0*	3.0*	3.0*	3.0*	3.0*	3.0*

*No growth; the 3 mm. represents the diameter of the disc of inoculum.

All strains produced normal mycelial colonies from 5° to 30°C. but at 33°C. the growth was hyaline and not white and consisted only of scant, submersed growth. Even though there were individual differences of 3 to 10 mm. in colony diameter by the strains between 10° and 30°C., between the groups A, B, and C the differences were small. In general, however, the averages for Group C were somewhat higher than for A and B.

The best growth by individual strain or by groups of strains was at 23°C., with some slight extension to 25°C., especially by Group C. Isaac (1949) noted that *I. albo-atrum* on Dox's medium grew best at 22.5°C. Above 25°C., all strains showed a rapid decline in growth rate.

It was observed that at temperatures of 10° and 15°C., all colonies produced a great abundance of black microsclerotia and chlamydospores, but there was a decrease in the numbers of these bodies with an increase in temperature, even though microsclerotia were still abundant in Group C at 20° to 25°C. Above 27°C. few microsclerotia and chlamydospores were found. Similar observations were made by Wilhelm (1948). Another noticeable effect of temperature was that at the lower and higher levels, below or above 20° to 25°C., the strains became progressively more indistinguishable from each other, except for the color imparted to the medium. All the characteristics by which the strains were distinguishable at 20° to 25°C. became more and more uniform and even though the gray, green, and black colors of the media beneath the colonies of groups A, B, and C, respectively, remained distinguishable, the difference was less pronounced. The tendency toward uniformity was more pronounced in group C, where the three strains became almost indistinguishable.

There was no growth of any of the strains at 35°C. When the discs of inoculum were subsequently transferred to fresh medium and incubated at 23°C., there was no growth; the mycelium obviously cannot survive exposure at 35°C.

RESPONSE OF THE 10 STRAINS TO DIFFERENT AMOUNTS OF VARIOUS CARBOHYDRATES

The ten strains were grown for 15 days at 23°C. on Czapek's agar medium containing 1, 3, 5, and 10 percent sucrose, dextrose, maltose or glycerine.

The results, listed in Table 26, show that only slight differences occurred in the growth of the colonies of the different strains at each of the carbohydrate levels. Colony growth was good at all levels of maltose and on media containing 3 and 5 percent sucrose, 1 percent glycerine and 5 percent dextrose. It was interesting to note that with concentrations of glycerine above 1 percent, colony growth declined rapidly.

Colonies grown on media containing 10 percent sucrose produced very few

microsclerotia and had a different superficial appearance. The texture appeared to be finer, and the white portions lacked lustre instead of being pure white (i.e. Sky-Colored White, Tone 1'). Cultures of V-2, V-6 and V-9 (group B) failed to produce the characteristic greenish pigment and, therefore, on this medium were almost indistinguishable from strains V-5 and V-10.

On media containing more than 3 percent glycerine, all strains except V-2, V-6 and V-9 (group B) produced a characteristic light orange color.

The mycelium of all colonies developed a grayish color at the 10 percent level of maltose; strains of groups A and C appeared similar. In fact, V-1 and V-7 (group A) produced a few of both black and brown microsclerotia. Strains V-2 and V-9 rarely produced black mycelium with this amount of maltose.

Cultures growing on media containing 5 percent and 10 percent of dextrose often developed black sectors. For example, three dishes of V-1, three of V-6, four of V-8 and one of V-3 displayed 3, 2, 4, and 2, 2, 4, and 2, 2, 3, 4, and 4 black sectors per dish, respectively.

As was noted in the case of temperature gradient above, the growth differences between the strains disappear or become modified in the presence of different carbohydrates. For example, V-2, V-6, and V-9 (group B) in the presence of 10 percent sucrose were similar to V-5 and V-10 of group C. The

Table 26 — The Effect of Various Levels of Different Carbon Sources in Czapek's Agar Medium upon the growth of 10 Isolates of *Vorticillium* sp.; 15 Days of Incubation at 23° C.

Carbon Source and Amount	Diameter of Colonies in mm. of the 10 Strains.									
	V-1	V-2	V-3	V-4	V-5	V-6	V-7	V-8	V-9	V-10
Sucrose										
1%	29.0	34.1	36.0	37.0	36.4	37.3	36.4	37.0	36.0	37.0
3%	66.1	64.0	64.4	65.0	65.7	66.3	66.0	65.1	66.3	67.1
5%	69.0	75.0	73.0	72.7	74.0	74.3	73.1	71.9	74.7	74.8
10%	46.1	49.0	48.0	48.7	49.0	42.0	48.3	47.4	49.1	49.0
Glycerine										
1%	63.0	65.0	71.0	64.0	63.0	62.9	63.1	63.6	66.0	63.0
3%	54.0	57.0	53.0	53.7	54.1	54.0	53.0	53.9	55.2	54.0
5%	24.0	28.0	22.0	23.0	24.2	23.9	24.0	24.1	27.0	24.3
10%	15.0	14.0	16.0	19.0	16.0	17.0	17.0	18.0	20.0	17.3
Maltose										
1%	60.0	60.0	59.0	61.0	60.0	59.0	58.7	61.0	60.0	60.0
3%	72.0	74.0	73.0	73.0	69.0	70.0	69.0	73.0	70.0	71.0
5%	79.0	81.0	80.0	79.0	80.0	79.0	79.0	81.0	81.0	79.2
10%	65.0	66.0	64.0	65.0	64.0	66.0	65.3	64.1	61.3	65.7
Dextrose										
1%	25.0	26.7	27.5	28.2	28.0	24.8	26.7	27.1	27.3	28.0
3%	34.0	35.0	35.9	35.2	35.0	34.7	35.0	35.1	24.5	35.7
5%	52.0	54.3	52.1	51.4	50.1	51.7	53.3	51.2	52.1	53.0
10%	41.0	42.2	40.2	42.7	42.3	41.1	41.9	41.0	42.0	42.1

¹Répertoire de Couleurs, published by La Société Française des Chrysanthémistes, et René Oberthür. 1905, page 5

production of a light orange pigment in the glycerine media by the members of groups A and C seems to indicate some similarity in the physiological activities of these groups.

RESPONSE OF 10 STRAINS TO DIFFERENT AMOUNTS OF VARIOUS SOURCES OF NITROGEN

The different sources of nitrogen tested were: peptone, sodium nitrate, ammonium nitrate, ammonium sulphate, casein and 1-asparagine, in amounts of 0.1, 0.5, and 1.0 percent. As before, the medium was Czapek's agar, the temperature 23°C., and the incubation time 15 days. The results are recorded in Table 27.

In general, there was good colonial growth by all of the 10 strains with all of the sources and amounts of nitrogen, except 1.0 percent 1-asparagine. At this concentration there were noticeable differences in the type of growth of strains V-2, V-6 and V-9 (group B). These not only lost their ability to produce the customary green pigment, but also failed to develop discernible black mycelium. Also, all of the cultures of all strains exhibited a persistently grayish aerial mycelium encircled by a definite peripheral white band, which at the end of 30 days was thick and cottony. Further, all of the colonies of all the strains not only produced black microsclerotia but produced them in a conspicuously different manner. On

Table 27 — The Effect of Various Amounts of Different Nitrogen Sources in Czapek's Agar on the Growth of 10 Strains of *Verticillium*.

Nitrogen Source and Amount	Diameter of Colonies Expressed in mm. of the 10 Isolates — 15 Days Growth at 23° C.									
	V-1	V-2	V-3	V-4	V-5	V-6	V-7	V-8	V-9	V-10
Peptone										
0.1%	73.0	74.0	73.0	71.0	60.0	73.0	69.2	74.0	72.6	74.0
0.5%	75.0	76.0	75.4	74.0	63.0	73.6	64.9	76.0	76.0	77.0
1.0%	78.0	78.2	77.9	76.6	67.0	78.1	66.5	80.0	79.3	80.1
Sodium Nitrate										
0.1%	74.0	73.0	75.0	74.0	73.0	73.2	74.0	71.0	73.0	74.1
0.5%	75.9	75.0	75.3	75.3	76.1	74.5	74.6	75.0	74.7	74.0
1.0%	77.0	76.0	75.0	74.0	75.0	77.0	73.0	75.6	76.1	77.3
Ammonium Nitrate										
0.1%	73.0	73.2	75.0	75.0	74.3	72.4	75.0	71.0	69.6	74.1
0.5%	75.0	74.5	76.0	73.0	77.1	76.0	74.0	74.0	74.6	75.3
1.0%	76.1	75.0	75.0	74.6	76.8	75.3	73.0	75.0	75.3	79.7
Ammonium Sulfate										
0.1%	76.2	74.2	76.0	76.3	74.1	73.1	75.3	74.0	74.0	76.3
0.5%	77.3	76.0	77.6	75.0	77.3	72.3	78.9	73.0	75.6	77.0
1.0%	79.3	79.0	74.3	75.6	76.6	79.0	81.7	77.0	78.1	80.1
Casein										
0.1%	74.0	69.0	75.0	74.3	75.4	75.0	74.4	67.3	75.0	74.2
0.5%	73.0	66.0	73.0	72.3	75.0	74.7	73.0	71.4	74.0	73.1
1.0%	61.0	61.3	62.0	60.0	61.3	71.1	69.1	63.6	59.3	64.0
1-Asparagine										
0.1%	64.0	63.7	64.0	65.0	63.7	64.2	62.9	64.0	65.0	64.3
0.5%	57.0	56.0	57.1	56.4	57.0	57.4	56.1	55.9	56.0	57.0
1.0%	34.0	34.0	33.0	37.0	47.0	33.0	34.1	35.1	33.9	34.2

the surface of the cultures, these bodies were formed in concentric rings, as by V-10 on the regular Czapek's medium. Beneath the surface, the microsclerotia were in small aggregations which gave the medium from below a stippled appearance.

CONCLUSIONS

A reasonably large number of isolates of the maple wilt fungus produced 10 distinguishable strains which could be separated into three groups when grown at or near the optimum temperature on media containing what are considered the optimum kinds and amounts of nutrients. With minor exceptions and variations, each strain reproduced its type upon repeated culturing on these media. When, however, these strains were subjected to temperatures lower or higher than the optimum and when the carbon and nitrogen sources were changed in kind and amount, not only did morphological and cultural changes result but also the strains tended to, or actually did, lose their identity, in whole or in part. Therefore, it appears that the 10 strains in question are probably not different species, subspecies or even varieties, but biotypes of a single species. This conclusion is in agreement with the opinion expressed by Rudolph [1931], Presley [1941] and [1950], and Wilhelm [1948] upon the basis of their studies of *Verticillium albo-atrum* derived from several crop-plant hosts.

Tests of the Pathogenicity of the 10 Strains and the Non-Matted White Variant

The purpose of this experiment was to determine whether the 10 strains in question could be distinguished on the basis of pathological criteria. Tomato plants and eggplants 6 to 8 inches tall growing in pots of five units each and also in flats, were inoculated with a spore suspension of each of the strains grown in Tochinai liquid medium for 21 days. The spore suspension was forced into the

Table 28—Pathogenicity Tests of 10 Strains of *Verticillium* and the Non-matted, White Variant, using Tomato Plants and Eggplants.

Inoculum	Date Inoculated	No. of Plants Used	No. of Tomato Plants Wilted			No. of Eggplants Wilted		
			12-1-52	1-6-53	2-2-53	12-1-52	1-6-52	2-2-53
V-1	9N/12N*	10/3**	5	8	8	2	2	3
V-2	9N/12N	10/5	5	8	9	3	3	4
V-3	9N/12N	10/3	6	8	8	2	2	3
V-4	9N/12N	10/3	5	7	8	1	2	3
V-5	9N/12N	10/3	5	8	8	1	3	3
V-6	9N/12N	10/3	5	9	9	1	2	3
V-7	9N/12N	10/3	6	9	9	2	2	2
V-8	9N/12N	5/2	6	9	10	2	2	2
V-9	9N/12N	7/3	5	7	8	1	3	3
V-10	9N/12N	8/3	5	7	9	1	2	2
Variant	9N	10/2	0	0	0	0	0	0

*Numerator shows inoculation for tomato plants, denominator for eggplants.

**Numerator shows number of tomato plants inoculated, denominator for number of eggplants inoculated.

stems of the plants just below the ground level by means of a hypodermic needle. Checks for each set of tests were inoculated by the same method using plain, sterile medium. Sections for culturing were taken from all plants exhibiting symptoms of disease. All of those inoculated with the 10 V strains yielded the fungus. Also 3 of the 10 tomato plants inoculated with the non-matted, white variant, which displayed a slight discoloration near the point of inoculation, yielded the fungus upon culturing.

The results of the experiment are listed in Table 28.

All strains except the non-matted, white variant were found to be systemic pathogens in both tomato plants and eggplants. No difference could be detected between the symptoms and the rate of progress of the wilt induced by the 10 strains on any of the plants inoculated. Usually, the first perceptible symptom of affected plants was a slight chlorosis of the lowest leaves, which later turned to a pale yellow and the margins became dark and curled inward. Finally, entire leaves would dry and fall. In some of the tomato plants, most of the leaves became flaccid simultaneously.

Other Comparisons of the 10 V Strains and the Non-Matted, White Variant

Experiments were conducted to find whether any physiological differences existed between the 10 V strains and the non-matted, white variant when grown in a liquid medium (medium H) and subjected to a 12-day shake period of incubation. All the cultures were tested for the production of polysaccharide and of thiourea, quantity of mycelium produced, and change in pH of the medium after incubation.

The results, as recorded in Table 29 show no significant differences for the

Table 29 — Comparative Tests of Isolates V-1 to V-10 and the Non-Matted, White Variant, with Respect to Change of pH of Medium, Amount of Mycelial Growth, and Production of Polysaccharide and Thiourea.

Isolate	No. of Flasks	pH of Medium after Shaking (initial pH 5.2)	Mycelial Weight (mg./ml.)	Amount of Polysac- charide	No. Flasks Showing Thiourea
V-1	9	6.5	227	1.7	1
V-2	8	6.7	229	1.6	1
V-3	7	6.5	232	1.6	1
V-4	6	6.7	219	1.9	1
V-5	7	6.5	231	1.9	1
V-6	7	6.4	234	1.7	2
V-7	7	6.7	228	1.7	1
V-8	8	6.5	236	1.9	1
V-9	8	6.2	230	1.4	2
V-10	7	6.8	227	1.5	1
White Variant	25	6.4	103	.3	0
Control	6	5.2	—	—	—

10 V strains in the amount of polysaccharide, the weight of the mycelium, or change in pH. All of these strains occasionally gave a positive test for thiourea. On the first run, only strains V-2, V-3, V-4, V-6, V-8 and V-9 produced this substance. In Table 29 both runs are grouped together. Thiourea was never produced by the non-matted, white variant, regardless of the period of incubation, whether in shake or still cultures. This variant produced less polysaccharide than any of the V strains.

Nuclear Situation in One of the Strains

The exact conditions responsible for the existence of biotypes are not known. Hansen (1938) and Hansen and Smith (1932) attributed genetic changes in species of the Fungi Imperfecti, including *Verticillium albo-atrum*, to the phenomenon of heterocaryosis — the presence in individual cells and individual spores of these fungi of at least two genetically distinct types of nuclei. Hansen (1938) is of the opinion that segregation can occur with greater ease in those fungi having more than one nucleus in a cell or spore. Since Hansen found that monoonidial isolates of *V. albo-atrum* produced more than one type of colony, he felt that spores of the fungus "will contain more than 1 nucleus and perhaps as many as 3 or 4 although this has not been definitely demonstrated." Hansen further reported that from 180 isolates of *V. albo-atrum* from various hosts and localities in California, 37 were distinct races. He did not formulate definite descriptions of these. On the assumption that they are different entities, such a multiplicity of races within this species is of interest and shows that adaptational biotypes must occur with rather high frequency in nature.

In order to obtain information regarding the nuclear condition in mycelial segments or spores of our *Verticillium*, cultures of strain V-10 were subjected to the Feulgen nuclear reaction described by Johansen (1940). After this treatment, nuclei in both mycelial segments and spores were stained a magenta color. Examination of over 75 preparations disclosed that 431 spores were mononucleate, 12 were bi-nucleate and 1 was tri-nucleate. Of the 344 mycelial segments examined, 319 had 1 nucleus, 22 had 2 nuclei, and 3 had 3 nuclei. It was also noted that a newly formed chlamydospore contained five definite, spherical, magenta-colored bodies. Should some of these nuclei be genetically different, the bi- and tri-nucleate conditions would perhaps partially explain the occurrence of different cultural strains of the maple wilt *Verticillium*.

THE ANTAGONISTIC ACTION OF A SOIL ACTINOMYCETE AND A BACTERIUM AGAINST VERTICILLIUM SP.

Antagonistic association between certain organisms on nutrient-agar plates has been known for more than half a century. Although it was conjectured that

this phenomenon was the result of the production of metabolites by the organisms concerned, it was not until recent detailed studies in the field of antibiosis that this speculation was verified. Several of the pure substances (antibiotics) isolated have been found to be highly effective as chemotherapeutic agents in the control of certain human diseases. Success in this field has stimulated the interest of phytopathologists as to the possible use of antibiotics against plant pathogens. Furthermore, advances in the treatment of vascular plant diseases with various chemotherapeutants (Howard 1941) suggest studies on the possible effectiveness of antibiotic substances.

Although numerous investigators have noted antagonistic properties of different soil actinomycetes, fungi, and bacteria against plant pathogens, none have reported the effectiveness of these agents against the maple wilt *Verticillium*.

The present investigations were undertaken after it was found in a preliminary experiment that *Bacillus subtilis* Cohn *EMEND.* Prazm. and an unidentified actinomycete displayed antagonistic properties against the maple wilt fungus when tested under laboratory conditions. Petri dishes containing potato-dextrose agar were separately inoculated at 3 or 4 points with the 2 organisms and allowed to incubate at room temperature for 48 hours, in order to allow for diffusion through the medium of the antagonistic metabolite produced by each organism. The surface of the agar medium was then atomized with a spore suspension of *Verticillium* V-10 and incubated at 20°C. for 21 days. The degree of antagonistic activity was determined by measuring the diameter of the clear zone around the colonies of the organism being tested. Ten replicates of the actinomycete were used and 12 of the bacterium.

A clear zone was observed around the colonies of both organisms in every case. As shown in Tables 30 and 31, the antagonistic metabolite produced by *B. subtilis* was either more potent or more diffusible through the medium than that produced by the actinomycete. In two dishes inoculated with this latter organism, the hyphae of the *Verticillium* were observed growing just below the entire surface of the medium, even beneath the colonies of the actinomycete. Also, after 4 or 5 weeks, the fungus grew up to the actinomycete colonies. This situation was never observed to occur in plates in which the bacterium was used, and the

Table 30 — Antibiotic Activity of Actinomycete Isolate Against *Verticillium* V-10.

Age of Culture (Days)	Av. Diameter of Clear Zone (mm.)	Growth of <i>Verticillium</i> Below Clear Zone
7	14	none
14	14	none
16	12.5	none
21	9.5	in 2 petri dishes
25	9	in 2 petri dishes
30	8	in 2 petri dishes

Table 31 — Antibiotic Activity of *B. subtilis* Isolate Against *Verticillium* V-10.

Age of Culture (Days)	Av. Diameter of Clear Zone (mm.)	Growth of <i>Verticillium</i> Below Clear Zone
6	15	none
8	15	none
15	13	none
19	14	none
22	14	none
25	14	none
30	12	none
33	12	none

indication is that the antibiotic substance produced by the bacterium had lasting effects.

Since it was found that *B. subtilis* produced a stronger antibiotic titre than the actinomycete, subsequent experiments were conducted using the bacterium alone, growing on a liquid medium of the following composition: yeast extract—5.0 gr.; dextrose—20.0 gr.; l-asparagine—1.0 gr.; K_2PO_4 —1.0 gr.; KCl —0.5 gr.; $MgSO_4 \cdot 7H_2O$ —0.5 gr.; $FeSO_4 \cdot 7H_2O$ —0.01 gr.; distilled water—1000 ml. The medium was dispensed into 250 ml. Erlenmeyer flasks, autoclaved and inoculated with uniform discs bearing the organisms. One group of flasks was placed in the shake machine and agitated for 9 days; the other group of flasks was allowed to stand for 15 days. At the end of these periods, the medium was freed of the organism by passing the cultures first through No. 50 Whatman filter paper and then *in vacuo* through a sterilized, ultra-fine, sintered-glass filter. The filtrate was then boiled for 20 minutes or autoclaved and used to test for the presence of an anti-fungal metabolite by using the paper disc method. These discs, approximately 10 mm. in diameter, punched out of No. 50 Whatman paper, were sterilized by dry heat and aseptically saturated with the filtrate to be tested. Three of these discs were then evenly spaced on Czapek's agar medium in each petri dish. At three points on the agar and between these discs, a small piece of agar covered with *Verticillium* was planted. Check plates were inoculated in a similar manner except that the paper discs were saturated with unused medium. Before each test, since the filtrate increased in alkalinity, the pH was adjusted to that of the unused medium. Five tests, with at least four replicates, were carried out, and in every case there was definite antagonistic action against the *Verticillium*.

In one experiment, strains V-1 and V-10 were used and both were definitely inhibited. It was noted, however, that V-1 was inhibited to a greater extent than V-10 (see Plate 11, petri dish 1).

In all check plates, the fungus grew over the entire surface of the medium and the filter paper.

Where the diffusible antibiotic was present, it was observed that the fungus reached its maximum growth in about four days and from that time on, the

growth of the colony continued toward the edge or center of the dishes. These colonies were usually characteristically ovoid and appeared to be growing away from the zone containing the antagonistic metabolite. Measurements made from the edges of the colonies to the edges of the paper discs ranged from 11 to 30 mm., with an average of 19.1 mm.

THE FUNGUS

A review of the literature, undertaken in an effort to obtain some information as to the identity of the maple wilt fungus, has disclosed that uncertainty exists as to what species of *Fusicillium* is responsible for this disease. When the maple wilt disease was first reported by Rankin in 1914, it was attributed to a fungus belonging to the genus *Acrostalagmus*. Although in 1918 Zimm referred to the fungus causing the disease reported by Rankin as *Fusicillium*, no specific identity was given to the organism.

Jagger and Stewart (1918) isolated a *Fusicillium* from several common plants, including maple. In their cross inoculations, results were as follows: the *Fusicillium* from barberry and eggplant infected maples, but that from potato did not; the *Fusicillium* from maple did not infect eggplants and barberry. They stated in one place (p. 16) that the cultures from eggplant, barberry and maple were indistinguishable, but the cultures from potatoes were markedly different from the cultures from the other hosts. In the summary (p. 19), they stated that the cultures of *Fusicillium* from potato, eggplant, barberry, salsify and several species of *Solanum* from the vicinity of Rochester, New York, were indistinguishable in rate of growth, macroscopic appearance of colonies and formation of sclerotia-like bodies, but the cultures from maple differed in the rate of formation of the sclerotia. They ended by stating that the fungus from Canadian potatoes appeared to be *F. albo-atrum* and the formation of sclerotia differed from that in the fungus common around Rochester, New York.

Although there has been agreement among various workers that the fungus responsible for maple wilt is a species of *Fusicillium*, there appears to be difference of opinion as to the exact species. Carter (1938, 1940, 1940a) and Dodge and Rickett (1948) referred to the fungus as *F. albo-atrum*, whereas Van der Meer (1926) and Ludbrook (1933) called it *F. Dahliae* Klebahn since the organism which they examined produced microsclerotia. Gravatt (1926), Pirone (1941) and Strong (1936, 1938) referred to the fungus merely as a species of *Fusicillium*.

Thus confusion prevails with regard to the name of the fungus causing maple wilt. For a review of the mycological situation and some possible enlightenment recourse was had to Saccardo vols. 4, 10, 11, 14, 16, 18, 22, and 25. In this discussion, it should be kept in mind that most, if not all, of the descriptions of the

species in this great work were made from the fungi as they occurred naturally on the various hosts and substrates, while our maple wilt fungus was available for comparison only in culture on Czapek's agar medium at 23°C., since as far as is known this fungus has not been seen growing or sporulating extramatrix on its natural host. The acceptability of any conclusions reached here depends entirely upon the validity of the foregoing condition of comparison. Furthermore, it should be kept in mind that, aside from the inadequacy of many of the descriptions in Sylloge Fungorum and the absence of an important number of definitive characters for a fungus of this kind in any event, much attention had to be paid to color manifestations and size of spores.

Accordingly, the 96 species and 7 varieties given in Saccardo were studied by an elimination process as follows.

1. *Species with no spore measurements given.* The descriptions of many of these 29 species are too brief or otherwise inadequate. Where details were given, color of the mycelium or shape of the spores eliminated the species from consideration as our fungus.

2. *Species with spores 1 to 4 u. long (19 species).* In the first place, only rarely has a spore of the maple wilt fungus been observed as small as 4 u. Further, none of the descriptions appeared to fit.

3. *Species with spores 4 to 6 u. long (23 species).* This spore size represents only the lower limits of the range of the maple wilt fungus and apart from many inadequate descriptions, there appeared to be no similarity to our fungus in any case.

4. *Species with spores over 12 u.* This group includes 3 species found on agaries and producing conidia 12 to 14 u, 18 u, and 25 to 30 u long—entirely above the range of the maple fungus, which was found to have only about 2 percent of the spores measuring as large as 12 u.

5. *Species with spores 6 to 12 u long (22 species).* A careful consideration of all the species in this group leads to the conclusion that only one description would fit the maple-wilt fungus and that is that of *Verticillium albo-atrum* Reinke and Berthold, even though Saccardo's description does not include reference to microsclerotia and chlamydospores which are produced by the maple wilt fungus on Czapek's agar medium.

A review of Reinke and Berthold's paper (1879), from which the description of *Verticillium albo-atrum* in Sylloge Fungorum was taken, disclosed that these two workers used the terms "Sclerotien" and "Dauernmycelien" interchangeably, with the former given preference, for structures their figures 2 & 3, Plate IX which are more or less similar to the microsclerotia produced by our fungus in culture. Whether or not these two workers found in rotting potato stems the

same well-developed microsclerotia and chlamydospores which our fungus produced *in vitro*, they at least had something of similar nature and function and it is strange that Saccardo's description does not mention them.

Klebahn (1913) isolated a *Verticillium* from diseased dahlia plants, which when grown on salep agar at room temperature produced microsclerotia. This he named *V. Dahliae* because *V. albo-atrum* did not produce microsclerotia under these same conditions.

In an excellent review of Klebahn's work, Rudolph (1931) expressed the opinion that microsclerotia were not of sufficient importance to be a specific criterion, since the conditions under which the fungi were grown may have been responsible for the difference. He obtained cultures of *V. albo-atrum* and *V. Dahliae* from the Centraalbureau voor Schimmelcultures at Baarn, Holland, and grew them both on Czapek's agar medium. He found that these fungi were morphologically indistinguishable, especially in that they both produced an abundance of microsclerotia on this medium and also in that they lost their ability to produce these structures after "considerable cultivation". It should be mentioned that Rudolph apparently gave no attention to the size of the spores.

One must agree with Rudolph that the production of microsclerotia by a *Verticillium* should not be considered as the sole criterion for differentiating species. It was shown above that the quantity and color of microsclerotia produced by the different strains of the maple wilt fungus on artificial media varied with different nutrient and temperature conditions and that two of the strains developed non-matted, white variants which were permanently without microsclerotia. It is possible that the same situation may occur in nature and that many of the species of *Verticillium* described in Saccardo may be biotypes of one species.

The fact remains that since the published description appears to be adequate and especially since the microsclerotia and chlamydospores in our cultures are fundamentally the same sort of structures called "Sclerotien" and "Dauermycelien" by Reinke and Berthold, the maple wilt fungus appears to be *V. albo-atrum* R. & B. Even though the species was originally described from potato, on the basis of all other information available at the present time, it would be unjustifiable to make a new species or a subspecies of the maple wilt fungus, especially since this fungus was successfully inoculated into plants of potato, tomato, cabbage, broccoli, eggplant, pepper and barberry, and incited a wilt in all but pepper and cabbage. Because of the vague and inadequate descriptions of the numerous species of *Verticillium* which appear in *Sylogae Fungorum* a monograph of the genus based upon morphological and physiological studies under controlled cultural conditions as well as host relations is sorely needed.

Isaac 1955 accorded specific rank to an isolate of *Verticillium* obtained from a Japanese maple *Acer palmatum* tree growing in the United States.* The organism which he called *Verticillium intertextum* sp. nov. did not induce wilt when inoculated into specimens of Japanese maples.

SUMMARY

Maple wilt caused by *Verticillium albo-atrum* Reinke and Berthold is a disease, which from the time of its recognition in 1914 has become increasingly prevalent on various species of maple used as ornamental trees.

The research undertaken was an endeavor to obtain information on the *modus operandi* of the disease. Emphasis was placed upon (1) factors influencing the initiation of the disease, (2) nature and properties of the toxic metabolites produced by the fungus, and (3) a comparative morphology and physiology study of 10 pathogenic strains of *Verticillium*.

Verticillium is capable of producing disease when introduced into the vascular system through wounds made at any point in the stem or root. No difference in severity of the disease was noticeable with either the agar-disc or the spore-suspension method of inoculation. Spread of the disease in the tree, as indicated by greenish discoloration of the sapwood and wilting of the foliage, was always upward from the point of inoculation. Inoculation of potted maple trees through the root system was successful, but disease could not be induced through the leaves.

Inoculations of field grown maples produced severe wilt during the period of May 14 to June 14, but the wilt produced was less severe from that time on, and none resulted from inoculations on August 31.

Trees inoculated at 2 to 5 points on the trunk displayed only slightly more severe wilt symptoms than did trees with only one inoculation, but more extensive sapwood discoloration occurred in trees with more than one inoculation. This weakens the belief that *Verticillium* wilt results from the plugging of the vessels.

Experiments on field grown trees showed that the disease was more severe (1) with the use of increasingly heavier spore suspensions, (2) with the addition of toxin to the spore suspension, and (3) with injury to the root system. Defoliation prior to inoculation did not favor the incidence of disease.

It has been shown that the incidence of disease was reduced with an increase of soil water.

Based upon the sapwood water content of diseased field trees made before and after inoculation, wilt symptoms and extensiveness of sapwood discoloration

*Isaac, I. 1955. A new Hyaline species of *Verticillium*: *V. Intertextum* sp. nov. Trans. British Mycological Society 38 (2); 143-156.

increased with a decrease in the water content of the sapwood and vice versa.

By means of a potato-isolation technique, it was proved that the maple wilt fungus can remain alive in the soil for at least two years.

Soil amendments such as ammonium sulphate, sodium nitrate, Nugreen, Milorganite, cyanamide, and a 6-8-7 "tree food" were applied in varying amounts around diseased, field grown maple trees. All except sodium nitrate gave some degree of control with ammonium sulphate affording the best.

Although the fungus grew well on most liquid and solid media, Czapek's agar medium was used to study certain physiological responses of the fungus. Tochinal's liquid medium was employed for spore production, and a Medium H was used in most of the investigations of the toxic metabolite.

V. albo-atrum produced a toxic filtrate which induced wilt in tomato and maple cuttings and potted maple trees. At least two components of this filtrate caused wilt; a polysaccharide stimulating stem wilt, and thiourea, which induced leaf wilt. The polysaccharide was always produced by all strains, but thiourea appeared in only 40 percent of the filtrates although produced by all the strains except the non-matted, white variant.

The toxic filtrate produced by strain V-10 was thermostable, gave negative reactions for urea, and inhibited liquid uptake in young tomato cuttings.

Of many chemicals tested, 17 showed fungistatic properties, and hydroquinone produced antidoting action against the toxic filtrate.

More than 150 isolates from wilted maples grown on Czapek's medium fell into 10 distinguishable strains, V-1 to V-10, on the basis of type and color of colony, color of the medium beneath the colonies, and relative abundance, grouping and color of microsclerotia and chlamydospores. Monoconidial isolates from each of these could be grouped as: *A* — V-1 and V-7; *B* — V-2, V-6, and V-9; *C* — V-3, V-4, V-5, V-8, and V-10. Certain strains produced a matted, white variant and a non-matted, white variant, the former of which reverted to the parent type, while the latter maintained its integrity regardless of the number of subsequent transfers.

Strains V-1 to V-10 exhibited morphological and cultural changes when grown on Czapek's medium at different temperatures, even to the point of becoming indistinguishable at certain temperatures above or below the optimum. With various sources and different amounts of carbon and nitrogen at the optimum temperature, the same was true in certain cases.

The ability to produce polysaccharide and thiourea and to cause disease was similar in strains V-1 to V-10, but lacking in the non-matted, white variant.

Tests for the Feulgen nuclear reaction disclosed that spores and cells in

mycelial fragments may contain more than one nucleus. This multi-nuclear cell condition may explain the existence of various strains of this *Verticillium*.

A soil actinomycete and a bacterium *Bacillus subtilis* each produced a metabolite which was antagonistic to strains V-1 and V-10 growing on Czapek's agar medium.

A study of the mycological literature upon the genus *Verticillium* indicates that the species inciting the wilt of maple is *V. albo-atrum* Reinke and Berthold, at least until a very comprehensive comparison of cultures of the species of this genus is made.

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